

DEVELOPMENT OF LIQUID CHROMATOGRAPHY TANDEM MASS
SPECTROMETRY INSTRUMENTATION AND METHODOLOGY FOR THE
INVESTIGATION OF BIOLOGICALLY ACTIVE COMPOUNDS

By

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This dissertation is dedicated to the memories of the men, women and children who were lost in the Great American Siege of September 11, 2001.

“If I have seen further, it is by standing on the shoulders of giants.”
-Sir Isaac Newton

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in
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DEVELOPMENT OF LIQUID CHROMATOGRAPHY TANDEM MASS
SPECTROMETRY INSTRUMENTATION AND METHODOLOGY FOR THE
INVESTIGATION OF BIOLOGICALLY ACTIVE COMPOUNDS

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A new versatile quadrupole ion trap mass spectrometer (QITMS) was developed with the ability to yield reliable capillary liquid chromatography tandem mass spectrometry (cLC/MS/MS) data. This was realized by adapting a ThermoFinnigan GCQ series QITMS to a home-built vacuum chamber. Access to the instrument's source code allowed the upgraded QITMS to be customized to meet the needs of any research project. Some of the key changes made to the QITMS include increasing the nominal mass range from 1000 to 1800 atomic mass units, altering the instrument's octopole circuitry to be under direct control of the GCQ system and devising a subroutine for dual MS/MS monitoring.

Integration of a low-flow microelectrospray ionization (μ ESI) source for the QITMS yielded a mass detection limit of 12 attomoles by infusion of the peptide angiotensin I. Additionally, the μ ESI source provided a direct coupling of the QITMS with cLC.

The intrinsic properties of liquid chromatography for providing sample concentration and analyte separation make the incorporation of cLC with MS a valuable means of further improving the sensitivity and selectivity of analyses. This was demonstrated by examining a standard peptide mixture, where angiotensin I was observed at a concentration of 2.5 nM by cLC/MS and at 10 pM when cLC/MS/MS was used.

To better understand the function of FMRFamide-like peptides (FLPs), extracts from the nematode *Caenorhabditis elegans* were examined via cLC/MS/MS to identify which of the putative peptides were expressed. Unfortunately, preliminary efforts were not successful in confirming the presence of any FLPs in the *C. elegans* extracts. However, the internal standard angiotensin I was observed within these complex samples at the level of 335 attomoles, validating the sensitivity of the QITMS and the cLC/MS/MS methodology for identifying analytes at trace levels in a biological matrix.

To assay the hepatotoxic metabolite norcocaine nitroxide and other N-oxidative metabolites of cocaine, a new LC/MS/MS method was established. Norcocaine nitroxide, a stable radical, was discovered to be reduced in the presence of acid, thereby requiring the mobile phase to be buffered to near pH 7 using ammonium acetate. The production of norcocaine nitroxide *in vitro* was confirmed by LC/MS/MS after incubation of 500 μ M N-hydroxynorcocaine in rat liver microsomes.

CHAPTER 1 INTRODUCTION

Development of new ionization methods, improved mass analyzers and advances in computer technology have combined to thrust mass spectrometry to the forefront of bioanalytical sciences. By combining separation methods such as liquid chromatography (LC)¹ or capillary electrophoresis (CE)² with tandem mass spectrometry (MS/MS),³ the informing power of mass spectrometry increases exponentially. This has led to the growth of mass spectrometry as a vital tool in the areas of toxicology, drug metabolism and proteomics.

The quadrupole ion trap mass spectrometer (QITMS)^{4,5} is currently one of the mass analyzers most widely used in bioanalytical chemistry. The versatility, rugged nature and relatively inexpensive cost of the QITMS make it a popular choice among experienced mass spectrometrists and novice end users. Additionally, the QITMS is able to perform multiple stages of mass spectrometry (MSⁿ)⁶ for increased structural information as well as measuring an ion's charge state via reduction of the scan rate^{7,8} for accurate mass determination. Furthermore, biologically relevant compounds such as peptides and proteins can be mass analyzed when the QITMS is interfaced with electrospray ionization (ESI)⁹ or with matrix-assisted laser desorption ionization (MALDI).¹⁰

The following sections in this introduction give brief historical perspectives and outline the scientific foundations on the quadrupole ion trap and electrospray ionization.

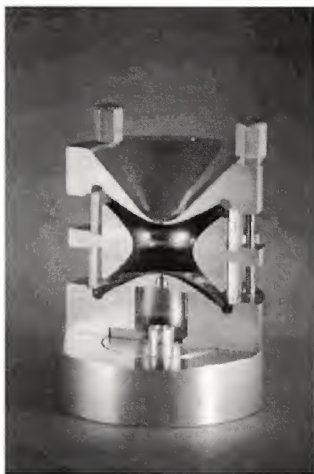
While the descriptions of these techniques are meant to be thorough, they are by no means exhaustive reviews on these topics and the reader is encouraged to consult the numerous articles on these techniques for more detailed information. This chapter concludes with an overview of the work described in this dissertation.

Quadrupole Ion Trap Mass Spectrometer

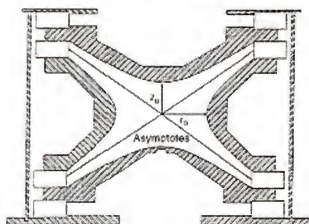
Background

The quadrupole ion trap (QIT) is one of the mass analyzers that has seen significant development in recent years. As first described in 1956 by Paul and Steinwedel,¹¹ the ion trap is derived via rotation of a cross section of a linear quadrupole (which was also described by Paul and Steinwedel¹¹) about one of its axes of symmetry, hence creating the three-dimensional quadrupole, as the ion trap is sometimes called. The quadrupole ion trap is comprised of three electrodes: two hyperboloid endcap electrodes sandwiching a rotationally symmetric ring electrode (Figure 1-1).

Early in its history, the QIT was utilized as a charged-particle storage device,¹² as a tool for the spectroscopic characterization of trapped ions¹³ and for examining ion-molecule reactions of light gaseous ions using an independent mass analyzer^{14,15} in addition to a mass spectrometer. The first instances of the QIT being used to measure an ion's mass-to-charge (m/z) ratio were by Fischer in 1959¹⁶ and later by Rettinghaus in 1967.¹⁷ These researchers used tuned circuits to measure the change in power on the endcap electrodes induced by the trapped ions as their frequencies moved into resonance with the auxiliary AC circuit. This nondestructive detection method, which is the basis



(A)



(B)

Figure 1-1: Cross sectional views of the QITMS.⁵ (A) Photograph of a QITMS and (B) schematic diagram of a QITMS along their plane of cylindrical symmetry. The ring electrode is positioned between the endcap electrodes. The axial and radial dimensions are indicated by z_0 and r_0 , respectively. [© John Wiley & Sons, Ltd. Reproduced with permission.]

of Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS), required complex circuitry and high vacuum ($<10^{-7}$ Torr) conditions and therefore QITMS research in this area was not further pursued until recently.^{18,19}

The first report describing the QIT as an analytical mass spectrometer was by Dawson and Whetten in 1968.^{20,21} Ions created by an electron beam introduced through the ring electrode were briefly stored then ejected via a DC voltage extraction pulse applied to one of the endcap electrodes. The ions were detected with an electron multiplier located directly behind the “drawout-cap electrode.” Although most of Dawson’s work on the ion trap was accomplished using a constant ratio of radiofrequency (RF) to DC voltage mode, operation of the QITMS without the use of DC voltage applied to the ring was described.^{20,21} By using RF/DC voltage scanning on the QITMS, ion peaks of higher resolution were obtained relative to operation in the RF-only (no DC) mode. However, only ions of a single m/z were stored and analyzed for a given ionization event when RF/DC scanning was employed, thereby making the recording of an entire mass spectrum inefficient. Nevertheless, Dawson’s research in the areas of the QITMS²⁰⁻²⁵ and quadrupole mass filter^{23,26} in the late 1960s and 1970s were pioneering and led to future advances in mass spectrometric instrumentation.

Maturation of the QITMS into an analytically useful instrument was realized through the accomplishments of Stafford and coworkers in the early 1980s.²⁷ Three key innovations were made during that period that led to the eventual commercialization of the QITMS: the mass-selective instability scan,^{28,29} the use of elevated pressures of helium,²⁹ and the stretched geometry of the QIT electrodes.³⁰

The first major step in bringing about the analytical development of the QITMS was utilization of the mass-selective instability scan.^{28,29} When the QIT is operated in the RF-only mode, ions stored within the ion trap are brought into resonance along the z-axis, or the axis of rotational symmetry, as they reach the $\beta_z=1$ boundary (see the Equations of Ion Motion section below). This resonance condition is accomplished by increasing the AC voltage on the ring electrode to the point where an ion's frequency is exactly one-half the frequency of the AC waveform applied to the ring electrode (i.e., the drive frequency). The ion's amplitude of motion increases until the ion either strikes an endcap electrode or, if holes are incorporated in the endcaps, the ion is ejected from the trap. Therefore, ramping the AC voltage on the ring electrode in a linear mode causes ejection of stored ions from the QIT in a sequential manner from low to high m/z .

The second breakthrough discovery was the effect of introducing helium gas into the quadrupole ion trap. During preliminary experiments coupling gas chromatography (GC) with the QITMS, it was observed that the mass resolution and the sensitivity of the instrument increased, particularly for high m/z ions, due to the elevated pressure in the ion trap from the helium carrier gas.²⁹ These improvements were attributed to removal of excess kinetic energy from the ions via collisional damping of the ions' motions to the center of the QIT prior to mass analysis. While unexpected, this finding had been first observed by Wuerker et al. in 1959¹² when "crystallization" of 32 charged aluminum particles was accomplished by elevating the background pressure within the ion trap to greater than 10^{-3} torr. Also, Dawson and Whetten²² observed that the storage of mercury ions became more efficient, both in terms of the total number of ions and the length of

time they remained trapped, with the addition of neon gas to an uncorrected pressure of 10^{-4} torr.

The third key development implemented by Stafford and coworkers was the stretching of the ion trap.³⁰ This final accomplishment was crucial to the commercialization of the QITMS²⁷ as it corrected the problem associated with certain ions being observed to shift to incorrect m/z assignments, even in the presence of internal standard ions. As described by a solution of the Mathieu equation (see below), the theoretical geometry for the QIT is $r_0^2 = 2z_0^2$. However, as a result of imperfections in the electrode machining, truncation of the electrodes and incorporation of holes in the endcap electrodes, higher-order (e.g., octopolar) fields overlap with the quadrupolar field.^{23,31} These higher-order fields cause ions to experience alternative motions to those described by the solution of the Mathieu equation for a pure quadrupolar field that may result in either premature or delayed ion ejection during mass analysis.³² In the case of the QIT developed by Stafford et al.,²⁷ the theoretical geometry ion trap had a small percentage of a negative octopolar field that had the effect of causing a delay in ion ejection relative to that in a pure quadrupolar field. The net result was that certain ions were ejected early while other ions were ejected late relative to the calibrant ions, which was later attributed to the gas-phase stabilities of the mass shifted ions.^{27,33} By symmetrically stretching the endcap electrodes further apart, a positive octopolar field was introduced into the QIT.^{27,30} The effect of this higher-order field was the elimination of the observed mass shifts of the ions investigated.²⁷ This can be attributed to the ejection of ions earlier than predicted in a pure quadrupolar field,³⁴ thereby reducing the

occurrence of ion dissociation due to collisions with the helium buffer gas during mass analysis.

After the introduction of the Finnigan ITD700 GC/MS in late 1984, a number of other instrumental advances have been attained including resonant excitation for MSⁿ,³⁵ resonant ejection for improved mass resolution,³⁶ automated gain control for preventing space charge effects³⁷ and external ion injection.³⁸ The last listed item is of particular importance for this body of work as it affords the coupling of electrospray ionization with the QITMS. Further discussion on such advances on the QITMS are discussed in the Basic Operating Principles of the Ion Trap section and in later chapters of this dissertation.

Equations of Ion Motion

As mentioned above, the quadrupole ion trap has rotational symmetry about the axis of revolution, typically defined in the literature as the z-axis. Because of this symmetry, the QIT is referenced using polar coordinates such that $x^2 + y^2 = r^2$, where r is the radial dimension in which the ring electrode lies. Hence, the dimensions of the ring electrode and endcap electrodes are referred to by the variables r_0 and z_0 , respectively, measured from the coordinate axes origin (i.e., the center of the ion trap) to the point normal to the origin on the electrodes, as shown in Figure 1-1B.

The motion of ions in a quadrupolar electric field is described by one possible solution to a second-order linear differential equation derived by the French mathematician Émile Léonard Mathieu in 1868.³⁹ Mathieu used vibrating elliptical membranes to mathematically describe the areas of stability and instability of the

produced standing waves.³⁹ From those regions of stability and instability Mathieu developed the general equation:

$$\frac{d^2 u}{d\xi^2} + (a_u - 2q_u \cos 2\xi)u = 0 \quad (1-1)$$

where u is the positional coordinate, a_u and q_u are reduced parameters (defined below) and ξ is the time-based variable.

With the boundaries of the quadrupolar field defined by the QIT electrodes, the motion of an ion within that electric field can be described by solutions of the Mathieu equation (Equation 1-1) by taking into account the force provided by the quadrupolar electric field on the ion.⁴ Derivation of the solutions to Equation 1-1 as it applies to the QIT is reported in other literature accounts^{4,40} and is not presented here. By assigning the positional variable to represent an ion's position in the axial ($u = z$) or radial ($u = r$) dimension and by setting the periodicity based variable as $\xi = \frac{\Omega t}{2}$, where Ω is the angular frequency of the waveform applied to the ring electrode in radians·sec⁻¹ and t is the instantaneous time in seconds, solutions for a_u and q_u that satisfy Equation 1-1 for the QIT are

$$a_z = -2a_r = \frac{-16eU}{m(r_0^2 + 2z_0^2)\Omega^2} \quad (1-2)$$

$$q_z = -2q_r = \frac{8eV}{m(r_0^2 + 2z_0^2)\Omega^2} \quad (1-3)$$

where e is the total charge on the ion in coulombs, U is the direct current (DC) amplitude in volts, V is the AC amplitude in volts zero-to-peak, r_0 and z_0 are the dimensions of the

ring and endcap electrodes, respectively, in meters (Figure 1-1B), and m is the mass of the ion in kilograms.

For ions subjected to a quadrupole field, the values for a and q do not explicitly indicate whether an ion has a stable or unstable trajectory within that field. Such information is provided by solutions to the Mathieu equation (Equation 1-1). An ion with a stable trajectory will have a finite value for u as ξ approaches infinity. However, solutions of Equation 1-1 that have infinite values for u indicate that an ion's motion will increase without limit until it is ejected from the trap or it strikes an electrode. In other words, for an ion to be trapped "indefinitely," it must have stable trajectories in *both* the axial (z) and radial (r) directions. This is shown by plotting solutions to the Mathieu equation in a_u/q_u space, also known as the Mathieu stability diagram.⁴⁰ Figure 1-2 displays the extended version of the stability diagram indicating where ion motion is stable in the r - and z -dimensions for values of a_u and q_u . Solutions of Equation 1-1 with finite values of u occur only where the r - and z -stable regions overlap. The largest overlap region is closest to the a_u/q_u origin and is referred to as the first stability region. This is the region in which the QITMS has traditionally operated and (to the author's knowledge) all ion trap mass spectrometers continue to operate. An expanded view of the first region stability diagram is shown in Figure 1-3.

Two key items exist concerning Figure 1-3. First, the stability region is bisected by curved lines termed iso- β lines.⁴⁰ These lines have values between 0 and 1 and indicate that a ion is stable in the z -direction if $0 < \beta_z < 1$ and in the r -direction if $0 < \beta_r < 1$. The value for β directly relates an ion's frequency of motion in the QIT to the angular frequency of the trapping field, Ω , by the following equation:

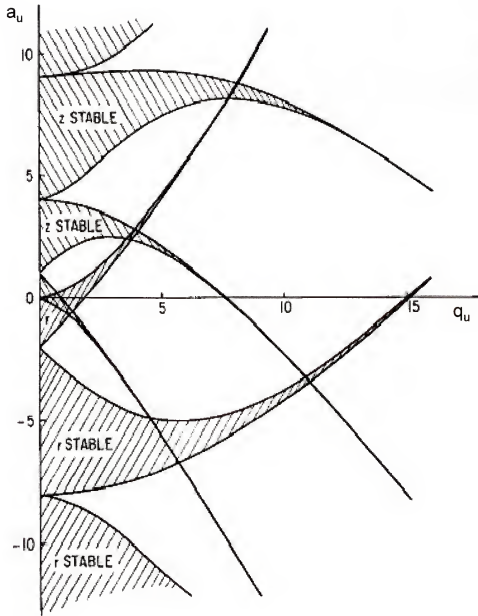


Figure 1-2: Extended Mathieu stability diagram.²⁰ Regions where the Mathieu equation yield finite solutions in the axial and radial dimensions are indicated by "z Stable" and "r Stable" hatched areas, respectively. The overlap region closest to the a_u/q_u origin is defined as the first stability region and corresponds to the conditions in which the QITMS is normally operated. [© American Institute of Physics. Adapted with permission.]

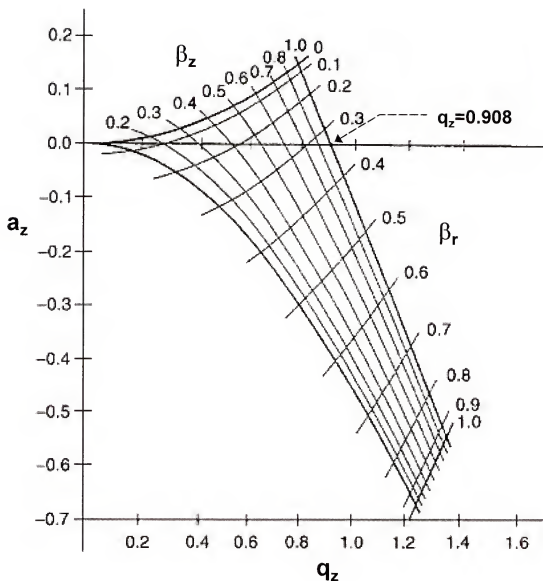


Figure 1-3: First stability region of Mathieu diagram.⁵ Normal operation of the QITMS is along the $a_z = 0$ line. For the mass-selective instability scan ions are axially ejected from the ion trap at the $\beta_z = 1$ resonance line, corresponding to a $q_z = 0.908$. [© John Wiley & Sons, Ltd. Adapted with permission.]

$$\omega_{u,n} = \left| \left(n + \frac{1}{2} \beta_u \right) \Omega \right|; \quad n = 0, \pm 1, \pm 2, \dots \quad (1-4)$$

where $\omega_{u,n}$ is the ion's angular frequency in radians·sec⁻¹. Therefore, for all a_u , q_u values along an iso- β_u line, an ion's frequency is identical in the u -dimension. For most purposes concerning the normal operation of the QITMS, the fundamental or secular frequency ($n = 0$) is the only ion frequency taken into consideration (see below). The other primary aspect of the stability diagram relates to the $\beta_z = 1$ line, particularly where this intersects the $a_u = 0$ axis (i.e., no DC potential applied to ring electrode). As discussed above, the mass-selective instability scan^{28,29} works by ramping the amplitude of the AC trapping field on the ring electrode until an ion's secular frequency, $\omega_{z,0}$, is one-half the drive frequency, Ω . As Equation 1-4 indicates, this occurs when $\beta_z = 1$. The ion at a frequency corresponding to $\beta_z = 1$ absorbs power from the trapping field and its orbit continually increases in the z -direction until it is ejected from the QIT. Interestingly, a subtle point about the operation of the ion trap in this mode is revealed by the stability diagram (Figure 1-3). Since an ion is still stable in the r -dimension at $\beta_z = 1$ (when $a_z = 0$ and $q_z = 0.908$, $\beta_r \cong 0.33$), the excursions of ion motion in the radial direction are small, thereby permitting the ions to escape the QIT through the endcap holes without striking the endcap electrodes. As mentioned previously, the motion of an ion within the QIT is dictated by the quadrupolar field imposed by the amplitude of the drive frequency on the ring electrode and the confining electrode surfaces. This ion motion in three dimensions is characteristic of a saddle as shown in Figure 1-4 (red trace).^{5,41} Upon close inspection, there are two independent frequencies of motion: the macromotion, representing the fundamental secular ion frequency ($n = 0$, Equation 1-4)

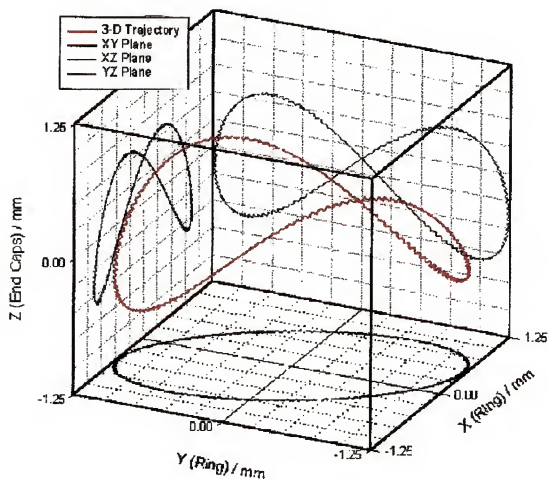


Figure 1-4: Three-dimensional representation of simulated ion motion in the QITMS.⁴⁰ The red trace displays the saddle-like secular motion with the overlapping low amplitude micromotion, representing the higher-order ion frequencies. Projections of the ion motion onto the two-dimensional planes (black traces) reveal the Lissajous figures commonly associated with an ion's trajectory within the QITMS. [© John Wiley & Sons, Ltd. Reproduced with permission.]

and the micromotion, where the higher-order frequencies ($n = \pm 1, \pm 2$, etc., Equation 1-4) are observed. The amplitude of the fundamental ion frequency is much greater than that of the higher-order frequencies (Figure 1-4). For this reason, only the fundamental secular frequency is taken into account for most practical operations of the QITMS.

For the remainder of this dissertation, ion motion/frequency is represented by values of q_z . The reason for using q_z for describing ions in the QIT is because of the simple relationship between an ion's mass-to-charge and q_z . Referring to Equation 1-3, q_z is inversely proportional to an ion's mass-to-charge, m/e (later represented by m/z , where z is number of charges on an ion). There is no such simple direct relationship between β_z and an ion's m/z . To determine an ion's m/z from β_z , the q_z value must first be calculated, which requires successive approximations from an expanded Taylor series equation.⁴⁰ Finally, since ions are mass analyzed via ejection through one of the endcap holes, which lies on the z -axis, it is logical to describe ions based on their axial motion as represented by the reduced parameter q_z .

Basic Operating Principles of the Ion Trap

There are three main stages of operation for the quadrupole ion trap mass spectrometer with external ion sources: ion formation and injection; ion storage and manipulation; and ion ejection for mass analysis. While past QITMS instruments formed ions within the trapping volume, nearly all currently available commercial ion traps use external ionization and ion transport to the QIT prior to mass analysis. One such external ionization source is electrospray ionization (ESI), which is discussed in the next section of this chapter.

Injection of ions into the QIT was once thought to be impossible.¹⁶ However, since the late 1980s a number of research groups have investigated the aspects of external ion injection into the QIT⁴²⁻⁴⁶ and have reported two major findings. For ions injected into the QIT to achieve stable orbits for extended periods of time (>1 ms), ions must have the proper amount of kinetic energy^{43,44,46} and ions must enter the trapping field during certain phases of the oscillating field.⁴²⁻⁴⁶ If these requirements are not met, the ions will either be stopped before entering the trapping volume or the ions will be accelerated until striking an electrode or ejected out of the QIT. Furthermore, even if the injected ions do meet the above stipulations, the excess ion kinetic energy required to penetrate the trapping field must be removed,³⁸ hence the use of helium as a motion dampening agent. For further information on the implications of external ion injection, particularly as it applies to ESI, the reader is referred to the dissertation of Scott Quarmby of the Yost group for full disclosure on this subject.⁴³

After ions have been injected into the QIT, all ion motion is dictated by the frequency, Ω , and amplitude, V , of the waveform applied to the ring electrode, unless the ions are acted upon by another external force (e.g., a supplementary AC waveform on the endcap electrodes, see below). The frequency of the waveform applied to the ring electrode is kept constant. Therefore an ion's motion and frequency is determined by the AC amplitude on the ring electrode, also termed the main RF voltage (since the waveform is in the radiofrequency region). These statements are represented mathematically by Equation 1-3, which indicates that $q_z \propto V$. However, the main RF voltage does not directly specify an ion's m/z without knowing the q_z value for that ion. Yet, by setting q_z to a constant value, an ion's m/z can be known for a fixed amplitude on

the main RF. Recall that the QITMS is normally operated in the RF-only mode (i.e., no DC amplitude, $a_z = 0$) and as such, the $\beta_z = 1$ boundary occurs at a $q_z = 0.908$. When the main RF trapping voltage is such that an ion's frequency is represented by a $q_z = 0.908$, then any ion with a m/z corresponding to the solution of Equation 1-3 will become axially unstable and be ejected from the QIT as described above for the mass-selective instability scan.²⁹ Because all other parameters in Equation 1-3 (r_0 , z_0 , Ω) are constant, the main RF voltage, V , at which an ion of given m/z has a corresponding $q_z = 0.908$ is readily predicted. Therefore, the main RF voltage is often represented by setting a certain ion m/z to a $q_z = 0.908$ and that m/z is termed the low mass cut-off (LMCO). By using the value for LMCO instead of the main RF amplitude, it is much easier to represent operational parameters among different QITMS instruments since ion motion at $q_z = 0.908$ is known and m/z is a more intuitive parameter for mass spectrometry than a voltage setting.

In simpler terms, when the main RF amplitude is at a fixed value, the motion of all ions within the QIT can be predicted. From Equation 1-3, the following simplification can be made: $[(m/z)q_z = \text{Constant}]$. In other words, there is a linear relationship between an ion's m/z and the Mathieu parameter q_z . Therefore, the relative position along the q_z axis (Figure 1-3) of any two ions within the QIT can be calculated from the equation

$$(m/z)_1 q_{z,1} = (m/z)_2 q_{z,2} \quad (1-5)$$

where the subscripts represent the ions' designations. A basic fact that can be inferred from Equation 1-5 is that ions of higher m/z are stored within the QIT at lower q_z and vice versa.

During the course of the QITMS scan function (i.e., the sequential events comprising the recording of a mass spectrum) the motions of the ions within the QIT are manipulated by the main RF amplitude, which is usually referred to in terms of LMCO or q_z of a certain ion's m/z . For a single-stage mass spectrum (MS), this usually involves only three discrete main RF voltage levels: ion injection and cooling; the MS first mass; and the MS last mass. For ion injection, the main RF amplitude is normally kept low so that injected ions can penetrate the trapping field and maintain low kinetic energies.^{43,44,46} After the ions have been collisionally cooled by the helium buffer gas to achieve stable orbits, the main RF is ramped to a LMCO corresponding to the first m/z to be recorded in the mass spectrum. Once the main RF voltage stabilizes, the linear ramp of the main RF amplitude results in the ejection of the ions from the QIT to be detected with an electron multiplier. For tandem mass spectrometry (MS/MS) experiments,³ in which ions are dissociated before final mass analysis, two additional steps are required. After ion cooling, the ion selected for dissociation is "ramped" to a high q_z value (e.g., $q_z = 0.83$) where ion isolation occurs (see below) followed by ramping the main RF amplitude down such that the selected ion is at a low q_z (e.g., $q_z = 0.25$) for ion dissociation (see below). The MS/MS data are then recorded from first m/z to last m/z in an analogous manner as described above by the main RF voltage ramp.

Ions within the quadrupolar electric field provided by the main RF waveform can be manipulated by another electric field, such as that applied to the endcap electrodes.

Indeed, ions were originally ejected from the QIT by a DC voltage pulse applied to one of the endcaps.^{20,21} In recent years, most QITMS instruments apply a supplemental AC waveform to the endcap electrodes to induce ion excitation or ion ejection when the ion's frequency matches that of the supplemental frequency. This method of ion stimulation, termed resonant excitation or ejection, was first accomplished by Fulford and coworkers⁴⁷ and later refined by Syka et al.⁴⁸ A sinusoidal waveform and its inverted complement (i.e., 180° out of phase) are applied to the endcaps during specific events during the scan function. The nature of this phased AC waveform is such that a dipolar field, which is constant within the trapping volume, is produced between the endcap electrodes (and overlaps the quadrupolar field from the ring electrode). The advantage of this dipolar field is that ions at or near the center of the QIT will experience the effect of this electric field but not that of higher-order fields (e.g., quadrupolar). The reason for this is that the field strength at the center of the ion trap is zero for electric fields greater than first-order. This is an important point given that the excursions of trapped ions from the center of the QIT are small after collisional cooling by the helium buffer gas.

The amplitude, frequency and time period of the supplemental AC waveform determine whether an ion gains enough kinetic energy to be ejected from the QIT (resonant ejection) or an ion undergoes enough collisions with the helium buffer gas to dissociate (resonant excitation). During the mass-selective instability scan,²⁹ in which the main RF amplitude is ramped, a supplemental waveform of several volts peak-to-peak (V_{pp}) and a frequency slightly less than half the drive frequency applied to the endcaps effects ion ejection through the endcap electrodes. Recall that at exactly one-half the drive frequency ions become axially unstable (z-dimension) since the ions' secular

frequency, $\omega_{z,0}$, corresponds to a $\beta_z=1$ (Equation 1-4). However, by providing an auxiliary AC field at a somewhat lower frequency, the ions will come into resonance with that supplemental field before reaching the $\beta_z=1$ boundary. Should the magnitude of that auxiliary field between the endcaps be sufficiently high, the excursions of the ions absorbing that supplementary field's power will grow without limit until the ions are ejected from the QIT. Resonant ejection of ions is analogous to creating a hole inside the stability diagram for which ions can escape. For mass analysis, resonant ejection is usually affected at a β_z value just less than 1, corresponding to a q_z somewhat less than 0.908 (e.g., $q_z=0.900$ on the Finnigan LCQ), sometimes referred to as q_{eject} .

During ion isolation for MS/MS experiments, resonant ejection is also used, but in a slightly different manner. Instead of applying a single-frequency waveform, the auxiliary waveform applied to the endcaps is a superposition of numerous frequencies corresponding to "all" frequencies of stored ions in the QIT, save the ion to be dissociated.⁴⁹ After ion isolation, dissociation of the selected precursor ion is effected by resonant excitation. Similarly to resonant ejection, an auxiliary AC waveform at a frequency corresponding to the storage q_z of the precursor ion, often called the q_{excite} , is briefly applied to the endcaps. The amplitude of the excitation AC waveform, sometimes referred as the "tickle voltage," is significantly less than that applied during resonant ejection, typically on the order of 1 V_{pp}. The kinetic energy of the resonantly excited ions is converted to internal energy via collisions with the helium atoms in the QIT. Once the ions have absorbed a critical amount of internal energy, covalent bond dissociation can occur, creating product ions. These fragment ions are subsequently mass analyzed yielding an MS/MS product ion spectrum.

There are certainly other analytically useful applications of the QITMS that can be discussed, such as mass range extension,⁸ improved mass resolution^{7,8} and MSⁿ.⁶ However, in the interest of brevity, the above descriptions of full-scan MS and MS/MS analysis on the QITMS should be sufficient for this introductory chapter. Other advanced QITMS operations are discussed later in this dissertation as necessary and the reader is encouraged to investigate the literature for detailed information on such advanced methods on the QITMS.

Electrospray Ionization

The inability to form intact gas-phase ions from nonvolatile molecules was a major limitation of mass spectrometry for many decades. Some ionization methods such as plasma desorption (PD)⁵⁰ and fast atom bombardment (FAB)⁵¹ showed some promise; however, these ionization methods had their limitations, particularly the difficulty in interfacing these ion sources with liquid chromatography. The ultimate breakthrough came when ions were produced in the condensed phase with subsequent liberation of the ions into the gas phase without the use of thermal energy, which is the mechanism of electrospray ionization.⁹ The following sections outline the development of ESI and its coupling to mass spectrometry and give an overview of the principles of ESI-MS.

History

The study of charged liquids is by no means a recent event. Indeed, Lord Rayleigh in 1882⁵² determined the maximum amount of charge a droplet of a given size could hold before becoming unstable (i.e., Rayleigh limit). Since that time much scientific research has been conducted in the area of charged liquids as it applies to many

disciplines;⁵³⁻⁵⁵ however, this discussion focuses on research events leading to the convergence of electrospray ionization with mass spectrometry.

In 1917, Zeleny examined the disintegration of glycerine droplets on the end of a capillary that was held at a high potential.⁵⁶ Photographs of the experiment showed that a cone of approximately 90° formed at the liquid surface and that a jet extended from the vertex of the cone towards the low potential surface. This work was later extended by Sir Geoffrey Taylor,⁵⁷ who developed the theories of Rayleigh to determine that the cone formed by the charged fluid inscribes an angle of 98.6° at the cone's apex. Taylor verified this hypothesis experimentally by using a soap film (Figure 1-5) and also by charging water submersed in a non-conductive fluid (transformer oil).⁵⁷ For his work, the liquid formation that develops during the electrospray process has been termed the "Taylor cone."

The first measurement of the size of ions formed from charged droplets is credited to Chapman.^{58,59} In 1937, Chapman used an ion mobility spectrometer, comprised of two parallel plates containing electrodes connected to an electrometer, to measure the mobility of electrified droplets.⁵⁸ Aqueous solutions containing various concentrations of different salts were charged with a platinum electrode and nebulized with a stream of air just before the inlet to the mobility spectrometer. From his examination of both positive and negative ions, Chapman concluded that the observation of the high mobility ions was attributed to a single charged particle with the adduction of a few water or air molecules. When the nebulized solutions were "aged" via angling the spraying device away from the spectrometer inlet, the data showed an increase in the low mobility ions with a

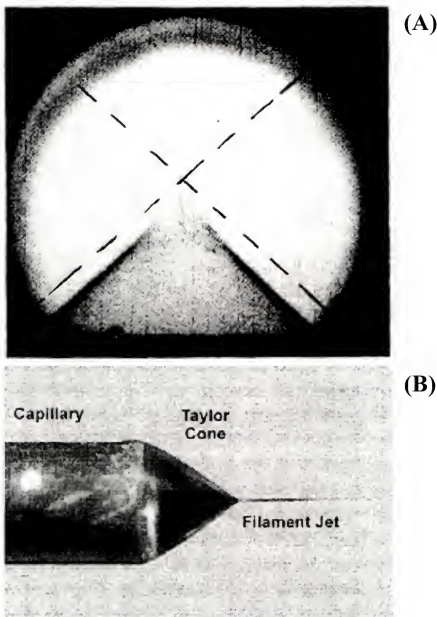


Figure 1-5: Images of electrosprays. (A) Photograph displaying the conical formation of an electrified soap solution. The broken lines inscribe a 98.6° angle about the liquid cone, as predicted by Sir Geoffery Taylor.⁵⁷ [© Royal Society of London. Reproduced with permission. Thanks to JSTOR for providing this material.] (B) CCD image of an electrospray, where the Taylor cone and a liquid jet emanating from the apex of the cone are clearly observed.⁶⁰ [© Elsevier Science. Reproduced with permission.]

concomitant decrease in the high mobility ions, which Chapman attributed to the “recombination, diffusion, or attachment (of ions) to slower carriers.” [p. 190 in ref. 58]

Thirty-one years later, Malcolm Dole and coworkers, in an attempt to more accurately measure the molecular weights of polymers, devised the idea to use electrospray ionization to create gas-phase ions via the evaporation of solvent to retain the intact polymer macroions.⁶¹ Interestingly, the Dole ESI source had many of the same basic characteristics of commercial ESI sources used today. A liquid solution was infused through the hypodermic needle held at high potential relative to the electrospray chamber, which was flushed with nitrogen. To transmit the gas-phase ions into the vacuum chamber, a nozzle-skimmer interface was employed that provided two stages of differential pumping between the ESI source at atmosphere pressure and the high vacuum region at 1×10^{-4} torr.⁶¹ However, because Dole et al. theorized (incorrectly) that only one or a few charges would remain on the polymers they electrosprayed, a drift tube with a Faraday cage detector and not a conventional mass spectrometer (e.g., a quadrupole mass filter) was employed to measure the polystyrene oligomers of nominal molecular weights of 51,000 and 411,000 atomic mass units (*u*).⁶¹ Despite the fact that Dole and coworkers never truly measured the ions’ *m/z* using a mass spectrometer, this pioneering work in the late 1960s and early 1970s established that intact gas-phase ions of nonvolatile compounds were formed,⁶¹⁻⁶³ including the protein lysozyme (14,600 *u*),⁶³ and it paved the way for the eventual development of ESI-MS (a term first coined by Dole).⁶¹

The first group to use a mass spectrometer to measure the *m/z* of ions generated from charged liquid droplets at atmospheric pressure was Thomson and Iribarne.⁶⁴ In

1979, a quadrupole mass filter was used to examine the ions generated from aqueous salt solutions by employing a high voltage (± 3500 V) electrode in close proximity (1 cm) to the liquid nebulizer probe. The introduction of this induction electrode increased the amount of ion current by two orders of magnitude over direct atomization of the salt solutions. This mode of ionization, which Thomson and Iribarne called field-induced ion evaporation,⁶⁴ produced water adducts of the electrolyte ions from the aqueous solutions. While the researchers mainly examined the ions produced by inorganic and organic salts, some mass spectra were obtained with neutral analytes (e.g., glucose) dissolved in the electrolyte solutions.⁶⁴ Such solutions yielded weak ion signals of the type $[M(H_2O)_n]X^\pm$, where M is the neutral analyte and $X^\pm$ represents the electrolyte cation or anion. The work of Thomson and Iribarne showed that mass spectrometry could be used to measure ions produced from the condensed phase; however, the first true use of electrospray ionization with mass spectrometry would not take place for several more years.

In 1984, Yamashita and Fenn at Yale University⁶⁵ and, independently, Aleksandrov et al. in the U.S.S.R.⁶⁶ advanced the research concepts of Dole by using a quadrupole mass filter and a magnetic sector mass spectrometer, respectively, to analyze the ions generated by ESI. These research groups unequivocally established that electrostatically charged droplets at atmospheric pressure could produce gas-phase ions free of solvent clusters from nonvolatile analytes in solution for mass-to-charge analysis without decomposition. A year later, the observation of multiply charged polypeptides was demonstrated by Fenn⁶⁷ and Aleksandrov and coworkers.⁶⁸ This led to the breakthrough work by the Fenn group in 1988 in which they successfully mass analyzed polyethylene glycol (PEG) oligomers (up to MW 17,500)⁶⁹ and biological

macromolecules (up to MW 39,830)⁷⁰ generated by ESI on a quadrupole mass spectrometer with a maximum range of m/z 1500. The ability to observe the macroions on a mass spectrometer with a limited m/z range is because these high molecular weight species take on multiple charges (Na^+ for PEGs and H^+ for biomolecules) when they are liberated into the gas phase by ESI. This demonstration of accurate molecular weight information (better than 0.2% relative error) for proteins, which required only trace amounts of sample (10^{-12} - 10^{-11} moles),⁷⁰ was the driving force for the biological mass spectrometry revolution.

The analytical development of ESI-MS, led by the research groups of Fenn,^{67,69,70} Henion,^{71,72} and Smith,^{73,74} proceeded rapidly in the late 1980s and early 1990s. The improvements in the ESI source design allowing high (200-1000 $\mu\text{L}/\text{min}$)^{71,75} and low (0.01-1.0 $\mu\text{L}/\text{min}$)⁷⁶⁻⁸² flow rates as well as higher tolerance to nonvolatile buffers (e.g., sodium phosphate)⁸³ are but a few of the major advances made over the past 14 years. Continued development and commercialization of ESI-MS have broadened the impact of mass spectrometry in many areas of scientific research and permitted the wide acceptance of mass spectrometry in the scientific community.

Theory and Fundamentals of ESI-MS

There are two primary theories describing the underlying process of gas-phase ion formation via electrospray ionization. The first theory was proposed by Dole⁶¹ who hypothesized that highly charged droplets, as they reached their Rayleigh limit (*vide infra*) during solvent evaporation, would undergo successive droplet fissions, or Coulombic explosions, until only the bare gas-phase ion remained. Iribarne and Thomson detailed the other ESI theory^{64,84} in which the electric field strength on the

charged droplets increases during solvent evaporation until the point where that field strength ejects ions on the droplet surface into the gas phase before the Rayleigh limit for the droplet is attained. The condition for which ion evaporation can preferentially occur over Rayleigh division is that the droplet's radius must drop below 8-13 nm.⁸⁴ In the current literature,^{85,86} these theories are termed the charged residue model (CRM) and ion desorption (or evaporation) model (IDM or IEM), respectively. Schematics representing the processes for these theories are shown in Figure 1-6.

While neither theory has been adequately proven, the IDM seem to be the favored mechanism for ESI over the CRM, as discussed by Fenn et al.⁸⁵ and Kebarle,⁸⁶ for low molecular weight compounds (<2000 *u*). The primary reason for preferring the IDM is that Iribarne and Thomson took into account the rate of ion evaporation based on the free Gibbs energy of solvation ($-\Delta G_{\text{sol}}$),⁸⁴ which relates to the chemical properties of an ion. Most notably, it has been well demonstrated⁸⁵⁻⁸⁹ that compounds that have a high surface activity (e.g., tetraalkylammonium salts) yield a higher sensitivity by ESI-MS than analytes with lower surface activity. If ions with a high surface activity are located on the charged droplet surface, the $-\Delta G_{\text{sol}}$ for that droplet is lowered and therefore the ion's surface activity increases the rate of ion desorption, leading to an increased ESI current. Dole's CRM does not take into account the physiochemical properties of analytes for ion formation, and hence the CRM cannot accurately describe differences in ion signal for electrosprayed compounds of similar mass at equal concentrations.

For macroions formed by ESI, arguments have been made to support both ionization mechanisms. Fenn⁸⁵ has endorsed the IDM since it is problematic (in his estimation) to explain how macromolecules can assume a Gaussian distribution of charge

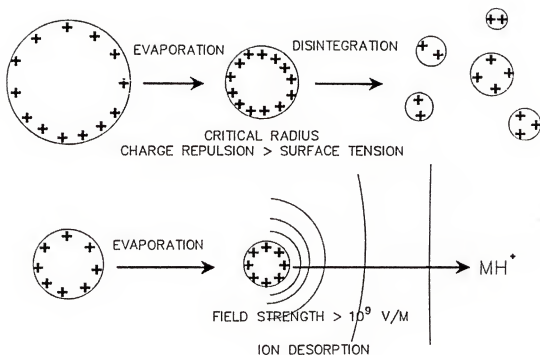


Figure 1-6: Schematics of the charged residue model (top) and the ion desorption model (bottom).⁸⁵ The CRM and IDM theories were originally proposed by Dole et al. and by Iribarne and Thomson, respectively (see text for details). [© American Chemical Society. Reproduced with permission.]

states based on changes in droplet mass and solution pH using the droplet fission mechanism of the CRM. Conversely, de la Mora⁹⁰ examined protein ions from aqueous solutions near neutral pH, where these proteins are in their native state, and determined that the number of charges on the protein ions, based on the observed ESI-MS, closely corresponded to the number of charges near the Rayleigh limit for droplets about the size of the protein, supporting the CRM.

Regardless of which theory best describes the ESI process, two postulates concerning ESI-MS are well documented. One, the early period of a charged droplet's lifetime is dictated by solvent evaporation leading to *asymmetric* coulombic fission as the precursor droplet's charge density approaches the Rayleigh limit (Figure 1-7).⁹¹ The resultant progeny droplets contain about 1.0 to 2.3% of the mass but 10 to 18% of the overall charge from the precursor droplet.⁹¹ Two, during the final stages of gas-phase ion production there may be no discernable difference between an ion desorbing from a droplet with several solvent cluster molecules and successive droplet fission to produce a single ion solvated by a couple of molecules.^{85,86,92} Based on these statements, both the CRM and IDM likely play a role during the ESI process.

Focusing on the analytical characteristics of ESI and how it is interfaced to a mass spectrometer, there are three general aspects involved in the ESI process: generation of a charged aerosol, formation of gas-phase ions, and ion transport from atmosphere to vacuum. Production of a stable electrospray is dependent on the strength of the electric field applied at the capillary outlet/liquid junction.^{57,81,86,92} Provided the force of the electric field is greater than the force due to the surface tension of the solution, the liquid surface will extend toward the counterelectrode in a conical shape called the Taylor cone

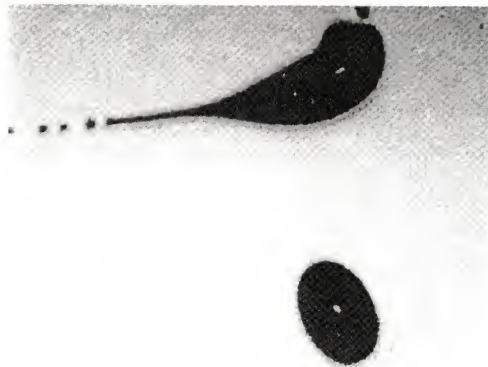


Figure 1-7: Flash shadowgraph displaying electrospayed droplets.⁹¹ The top droplet is undergoing asymmetric coulombic disintegration as it approaches the Rayleigh limit. The progeny droplets are approximately 1.0-2.3% the mass of the parent droplet; however, the product droplets retain 10-18% of the charge. The direction of droplet motion is from top to bottom. [© American Institute of Physics. Reproduced with permission.]

(Figure 1-5). A filament of liquid protrudes from the apex of the Taylor cone that eventually decomposes into individual charged droplets. These charged droplets disperse due to space charge effects and undergo Coulombic fission (Figure 1-7) to eventually produce gas-phase ions as discussed above.

Upon closer inspection of the ESI process on the molecular level, production of a stable Taylor cone requires an excess amount of charge of a certain polarity at the surface of the liquid as it exits the capillary tubing.^{67,86,92,93} Even when an electrolyte is present in the solution, the net charge of the liquid is zero. In order to produce an excess amount of charge on a liquid surface, an electrochemical reaction must take place.^{86,92} A low potential applied to the capillary (assume for this discussion that the capillary is metallic) will polarize the liquid, yet the net charge of the solution will remain zero. If the voltage applied to the metal capillary is increased, then the component(s) within the liquid having the low redox potentials will undergo an electrochemical reaction. In the case of positive ion electrospray the capillary acts as the anode, where oxidation takes place, resulting in the production of excess positive charge on the liquid surface. When the charge density and the electric field are sufficient to overcome the liquid's surface tension, the solution is forced downfield toward the counterelectrode, where reduction occurs to retain charge conservation, as depicted in Figure 1-8. Production of negative ions by ESI is realized by switching the polarity of the voltage applied to the capillary.

To reiterate, the major premise of ESI is its ability to form gas-phase ions from the condensed phase. After the production of charged droplets has taken place, there are two principal means by which analytes become gas-phase ions based on their chemical nature. One involves "preformed" ions existing as free electrolytes or produced either

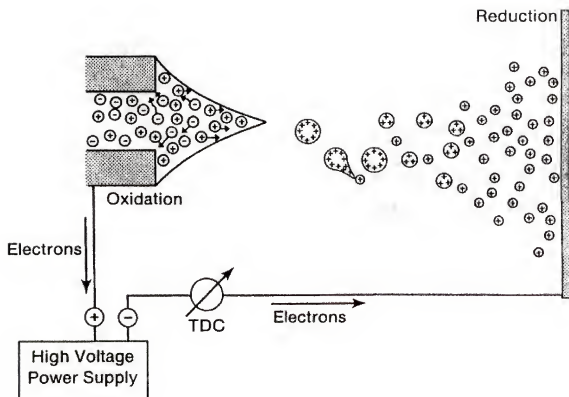


Figure 1-8: Schematic of the electrochemical processes that occur during positive ion ESI.⁸⁶ The high potential applied to the metal capillary causes oxidation at the liquid/metal interface, thereby enriching positive charges on the surface of the liquid. Charge balance is maintained by reduction at the counterelectrode (e.g., heated capillary inlet, skimmer, mass analyzer electrode, etc.). [© John Wiley & Sons, Ltd. Reproduced with permission.]

by Brönsted/Lowry acid-base chemistry or by a redox reaction at the ESI capillary.⁹⁴ The ions having the same polarity as the droplet's net charge will be drawn to the surface of the droplet and, after sufficient solvent evaporation and/or droplet disintegration, the ions will be ejected into the gas phase.^{86,92} The other ionization mechanism concerns polar neutral analytes. These uncharged species migrate by Brownian motion until nearing the surface of the droplet where the excess charge is retained. An analyte molecule can then become charged via a Brönsted/Lowry acid-base reaction (e.g., protonation) or a Lewis acid-base reaction (e.g., Na^+ attachment). Once charged, the analyte ion will be "anchored" to the droplet surface for eventual liberation into the gas phase.^{86,92}

Introduction of the gas-phase ions from the atmospheric region into the high vacuum region for mass analysis is accomplished by a series of physical elements with small orifices (e.g., 0.5 mm). The purpose of having multiple barriers between the ESI source and the mass analyzer is to allow several stages of differential pumping, which efficiently steps down the pressure from 760 torr to 10^{-5} - 10^{-9} torr, depending on the number of regions of pumping and the size of the vacuum pumps. In most commercial instruments produced today, there are two basic ESI interface designs: the nozzle-skimmer and the heated capillary-skimmer. For the ESI interfaces that use a nozzle-type counterelectrode, droplet desolvation is assisted by employing a countercurrent flow of nitrogen gas,⁶⁷ which is sometimes heated to improve the desolvation efficiency (especially at high liquid flow rates).^{74,95} The heated capillary design relies on the addition of thermal energy to assist droplet desolvation at atmospheric pressure.⁹⁶ Additionally, all modern ESI interfaces employ a coaxial nitrogen gas flow at the ESI capillary to increase desolvation efficiency.⁷¹

Ions are driven from the ESI source toward the mass spectrometer by means of an electric potential gradient as well as a pressure differential. Once the electrosprayed ions reach a region of lower pressure (e.g., after the heated capillary), the gas and entrained ions experience supersonic jet expansion.^{97,98} In this expansion region, called the silent zone,⁹⁷ the number density of gas molecules decreases as the ions axially transverse this region, thereby causing a rapid decrease in the ions' kinetic energy.⁹⁸ This cooling effect can cause ion-molecule clustering with residual solvent molecules and is the reason why Dole et al.,⁶¹⁻⁶³ and Thomson and Iribarne⁶⁴ observed low mobility ions and cluster ions generated at atmospheric pressure. To counter this effect, a sufficient acceleration potential between the inlet electrode and skimmer "warms" the ions and effects ion declustering.^{66,67} The skimmer electrode samples ions from the silent zone before reaching the Mach disk (Figure 1-9), where the ions' velocities coincide with the speed of sound at the local pressure. The equation predicting the distance between the nozzle (or heated capillary) exit and the Mach disk is given by

$$x_M = \frac{2}{3} D_o (P_0 / P_1)^{0.5} \quad (1-6)$$

where D_o is the orifice diameter of the nozzle (heated capillary), P_0 is the pressure in the region before the jet expansion and P_1 is the pressure in the region containing the Mach disk. It is important to position the ion collimating skimmer at a distance less than that predicted by Equation 1-6 to prevent ion sampling losses due to scattering beyond the Mach disk. Once beyond the skimmer, ions traverse the high vacuum region by means of electric or magnetic fields.

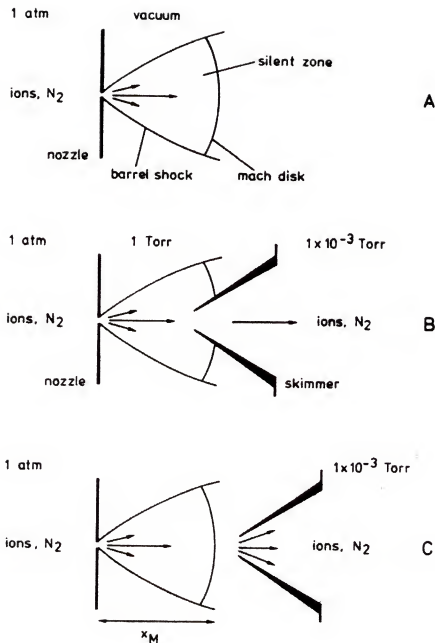


Figure 1-9: Schematics of the supersonic jet expansion region.⁹⁷ (A) Representation of the jet expansion that gaseous ions and neutrals experience when passing through a pressure differential separated by a small orifice. (B) The nozzle-skimmer setup often employed in modern ESI interfaces, where the ions are sampled before the Mach disk as to take advantage of the beam-like movement within the silent zone. (C) Arrangement of the skimmer beyond the Mach disk where ions have undergone significant scattering. [© John Wiley & Sons, Inc. Reproduced with permission.]

As a final key aspect, the observed signal in ESI-MS is mainly independent of the liquid flow rate delivered to the ESI source.⁹⁹ In other words, ESI-MS acts as a concentration-sensitive device,¹⁰⁰ even though a mass spectrometer is a mass sensitive detector.⁹⁹ There are two hypotheses for this phenomenon.⁹⁹ One, at a constant transmission efficiency of ions from atmosphere to vacuum, an increase in the liquid flow rate to the ESI emitter is offset by a concomitant decrease in the ionization efficiency. Two, the ESI efficiency remains constant at all flow rates; however, an increase in the ion flux at higher flow rates corresponds to a proportional decrease in the ion transmission efficiency to the high-vacuum region. Whichever the case, this intriguing characteristic of ESI-MS offers two distinct advantages: flow splitting before the ESI-MS for using independent detection or fraction collecting, and electrospraying at low flow rates, each without compromise in detection efficiency. The later of these advantages is particularly important analytically, as low flow ESI-MS provides superior mass sensitivity and the ability to couple ESI-MS to capillary separation techniques.^{77,80,101,102}

Low Flow ESI-MS

The ability to generate a stable electrospray is dependent on the magnitude of the electric field at the ESI needle to overcome the surface tension of the liquid (*vide infra*).^{57,81,86,92} At a fixed voltage, the electric field strength applied to the fluid is inversely dependent on the inner diameter (I.D.) of the capillary tubing delivering the liquid.⁹² Therefore, as the I.D. of the ESI capillary is reduced, a lower potential is necessary to establish a sufficient electric field to generate a stable electrospray.

In addition to the electric field strength at the ESI emitter, maintaining a stable electrospray requires that the liquid delivered to the ESI capillary have a minimum linear

velocity.^{81,103} This experimental requisite is the reason that conventional ESI sources do not function below certain flow rates. As empirically demonstrated by Covey and Balogh¹⁰⁴ and by Bateman, White and Thibault,¹⁰⁵ the ESI emitter becomes “starved” and the ESI-MS signal fluctuates erratically below some threshold flow rate for a given capillary I.D. (Figure 1-10). Based on experiments conducted by Vanhoutte and coworkers,¹⁰³ it was concluded that a flow rate corresponding to a linear velocity greater than approximately 1000 mm/min. at the ESI emitter was required to sustain a stable electrospray. Since linear velocity is inversely proportional to the square of the inner diameter of the tubing, employing smaller I.D. capillaries as ESI emitters provide the necessary linear velocities to achieve stable electrosprays at submicroliter per minute flow rates.

There are two basic types of ESI emitters used for nanoliter per minute ESI-MS. The first is the design of Wilm and Mann^{81,82} in which borosilicate glass capillaries are tapered to an I.D. of 1-2 μm and coated with a conductive metal (e.g., gold) at the ESI emitter end. This source design has been termed nanoelectrospray ionization (nESI)⁸² since it operates at flow rates from 10-40 nL/min. The nESI source operates at such low flow rates not only as a result of the extremely small capillary diameter, but also because the liquid flow is maintained by the applied high voltage at the emitter tip. The other general low-flow ESI source, termed microelectrospray,⁷⁷ uses nonconductive fused silica capillaries that are sharpened at one end by chemical, thermal or mechanical means to inner diameters on the order of 3-20 μm .⁷⁶⁻⁸⁰ To generate an electrospray, the high voltage is applied either at metallized tips^{78,79} or at a liquid junction^{76,77,80} (e.g., stainless steel union) upstream of the microelectrospray ionization (μESI) emitter tip. The major

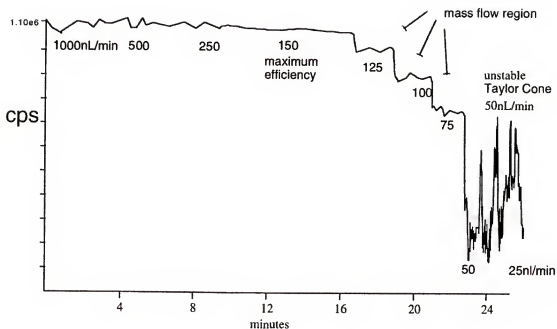


Figure 1-10: ESI-MS signal as a function of flow rate using a 20 μm I.D. capillary.¹⁰⁴ When the linear velocity, which is proportional to volumetric flow rate and inversely proportional to the square of the capillary I.D., drops below a certain value the ESI-MS no longer acts as a concentration-sensitive detector, but rather as a mass-sensitive device. At still lower linear velocities, the ESI emitter becomes deprived of sufficient liquid to maintain a stable electrospray resulting in a highly erratic ESI-MS signal. [Reproduced with permission from Thomas Covey.]

difference between the nESI and μ ESI sources is that pressure is the driving force generating liquid flow in the microelectrospray source. Also, the larger I.D.s employed with μ ESI emitters require higher flow rates, typically 50-500 nL/min.

While the nanoelectrospray source has been shown to be highly efficient, with up to 10% of analyte ions transmitted from solution into the vacuum system⁸² as opposed to $\leq 0.1\%$ with conventional ESI sources,⁸⁸ the nESI source is limited to examining the solution contained within the borosilicate emitter at any given time. At present, the nESI source design has not been adapted to examine different solutions without interrupting the nESI-MS experiment to change the sample. However, with the μ ESI source, changing solution compositions is not problematic. μ ESI emitters are composed of fused silica, which has greater tensile strength than borosilicate glass and is easily adapted to pressure connectors (e.g., unions and tees). These pressure connectors are necessary for mating fused silica tubing to liquid delivery units such as LC pumps and CE power supplies. Therefore, the μ ESI source design has been adopted for use with capillary separations.^{77,80,101,102} Additionally, the flow rates employed with capillary liquid chromatography (cLC)^{77,80} and capillary electrophoresis^{101,102} are compatible with those used on the μ ESI sources and obviates the need for a sheath liquid flow employed on conventional ESI sources,^{74,106,107} which lowers the sensitivity and degrades chromatographic resolution by diluting the analyte band as it elutes.

Dissertation Overview

The premise of this body of work was to develop new, versatile and sensitive LC/MS/MS instrumentation and methodology for the purpose of examining bioanalytical

problems. Chapter 2 presents the initial results of the μ ESI interface on the ThermoFinnigan LCQ and ITMS quadrupole ion trap mass spectrometers followed by an extensive description of the development and optimization of the new research-grade QITMS. Incorporation of a capillary liquid chromatography system to the μ ESI-QITMS instrument is the focus of Chapter 3. Details outlining the setup, capillary column design and overall instrument sensitivity with biological sample mixtures are presented. Chapter 4 describes the application of the cLC/ μ ESI-QITMS system towards the investigation of neuropeptides. Examination of extracts from the nematode *Caenorhabditis elegans* for FRMRamide-like peptides by cLC/MS/MS methodology is demonstrated. Development of a new LC/MS/MS method for the characterization of the hepatotoxic norcocaine nitroxide *in vitro* is the focus of Chapter 5. An N-oxidative metabolite of cocaine, norcocaine nitroxide is a stable organic radical, which had yet to be characterized by LC/MS/MS. Chapter 6 overviews the advantages, disadvantages and conclusions from this research effort, which finishes with some possible future research directions, both in terms of instrumentation development and bioanalytical applications.

CHAPTER 2 DEVELOPMENT OF THE QITMS WITH MICROELECTROSPRAY IONIZATION INTERFACE

Preliminary Microelectrospray Ionization Experiments

A Finnigan LCQ quadrupole ion trap mass spectrometer was used as the platform for conducting the early development and sensitivity experiments of the μ ESI interface. This commercially available QITMS was specifically designed to operate with an ESI source; hence, only minimal changes to the instrument configuration were necessary to execute the initial μ ESI-MS experiments on the LCQ. The high-flow ESI source probe head was removed and replaced with the μ ESI interface. The μ ESI source assembly consists of three parts: a Delrin probe, an XYZ translation stage and an aluminum mounting plate. The Delrin probe is used to hold the μ ESI emitter in close proximity to the LCQ heated capillary inlet, which serves as the counter electrode. The position of the μ ESI emitter is adjusted with respect to the heated capillary via the XYZ stage that secures the Delrin probe. The translation stage is mounted to the aluminum interface plate, which is bolted to the LCQ vacuum manifold in the position where the conventional ESI source was removed. *Note that this μ ESI source design defeats the high voltage protection circuit of the LCQ and care should be taken when using this setup.*

As discussed in Chapter 1, to generate a stable electrospray at low flow rates (<1 μ L/min), the electrospray ionization emitter must be tapered to a smaller inner diameter

than that used on conventional ESI sources.⁸¹ The μ ESI emitters are constructed from fused silica capillary tubing. For all μ ESI-MS data presented in this dissertation, 365 μ m O.D., 50 μ m I.D. fused silica tubing was used. A Sutter P-2000 laser micropipette puller (courtesy of the Dr. Tan group) was employed to taper the fused silica to a fine tip. This device creates two such μ ESI emitters when a sufficient length of tubing is used. Early attempts at pulling μ ESI emitters formed capillaries that were sealed at the tapered end; therefore, the narrowed end was opened with a quartz cutter with the assistance of an optical microscope. After refining the laser puller's parameters, the created μ ESI emitters had I.D.s approximately 4-8 μ m and these μ ESI needles were used without further modification. The parameters selected to use on the Sutter laser puller are given in Chapter 3.

The distal end of the tapered fused silica capillary was connected to a PEEK tee, which served as the liquid junction for the introduction of the sample solution and for the application of the high voltage for establishing the electrospray via a stainless steel (SS) or platinum electrode. To generate a stable electrospray for positive ion analyses, a positive voltage between 1.5 and 2.5 kV was applied at the liquid junction. In contrast to the LCQ ESI source, the μ ESI source does not use pneumatic assistance for droplet desolvation. Additionally, the temperature of the heated capillary, the inlet to the mass spectrometer, was lowered to 150-175° C as opposed to 200-225° C on the conventional ESI source. An optical device was not employed to position the μ ESI emitter; however, using differential measurements with the XYZ stage, the estimated μ ESI emitter/heated capillary distance was 1-3 mm. Samples to be analyzed by μ ESI-MS were prepared in

methanol:water mixtures acidified with formic acid (0.10% v/v) unless otherwise noted. A Harvard Apparatus Model 22 syringe pump was used to infuse the sample solutions at flow rates between 100 and 300 nL/min.

Reserpine at a concentration of 100 pg/ μ L was infused at 0.1 μ L/min. to yield the μ ESI-MS data in Figure 2-1A. A comparative mass spectrum *of the same solution* infused at 3 μ L/min employing the standard ESI source is given in Figure 2-1B. The experimental conditions were kept the same with the exception of the ESI source parameters (e.g., ESI high voltage) to directly compare the results of the two ESI interfaces. The first item of note is that the signal intensities for the $(M+H)^+$ of reserpine at m/z 609 are equivalent within experimental error for both ESI sources, again demonstrating that ESI-MS acts as a concentration-sensitive device.^{99,100} Another observation is the increased chemical noise in the μ ESI mass spectrum (Figure 2-1A), possibly due to the improved ionization efficiency of trace contaminants at lower flow rates.⁸² Finally, these data in Figure 2-1 were acquired for the same length of time (1.72 sec) with a constant ion injection time (15 ms); however, the μ ESI-MS data consumes 30 times less sample than the high-flow ESI-MS data (0.29 vs. 8.6 pg). Based on these results, μ ESI clearly provides the ability to acquire high quality mass spectra while consuming trace amounts of sample.

Shortcomings of the ITMS

In 1985, Finnigan offered its first research-grade quadrupole ion trap mass spectrometer, the ITMS. It was a highly versatile platform that allowed the user to vary every aspect of the instrument, both hardware and software. In the years that followed, a

number of analytical developments were made using the ITMS instrument, including resonant ejection³⁶ and external ion injection.³⁸ Nevertheless, the ITMS was not designed to analyze high mass compounds typically examined by ESI-MS. While several research groups interfaced ESI to the ITMS¹⁰⁸⁻¹¹² and they were able to analyze high m/z ions by extending the nominal m/z range of the ITMS (650 u) via lowering the resonant ejection frequency,⁸ these groups did so at the cost of mass resolution and mass accuracy. These limitations are again demonstrated by the data presented below.

The original goal of the doctoral research was to investigate the conformations of protein ions in the gas phase via hydrogen/deuterium (H/D) exchange reactions with D₂O in the ion trap. The μ ESI source was developed to be able to monitor these reactions on the time scale of hours on limited protein samples. After successfully producing ions via μ ESI on the LCQ, the μ ESI interface was adapted to fit the modified Analytica ESI source on the ITMS instrument originally developed by James Stephenson.¹¹³ The mass spectra of various standards acquired on the ITMS using the μ ESI interface were by inspection equivalent in quality and sensitivity to the μ ESI-MS data obtained on the LCQ. The subsequent step was to attempt H/D exchange reactions on a well-characterized protein, myoglobin.¹¹⁴⁻¹¹⁷

One of the primary advantages of ESI is the formation of multiply charged ions for compounds containing numerous basic or acidic sites such as proteins and oligonucleotides.¹¹⁸ This fundamental property of ESI allows the analysis of high mass ions with mass spectrometers of limited mass range.^{69,70,118} The ITMS was designed with a nominal m/z range (defined here as the maximum m/z observed if ions are ejected at $q_z=0.908$) of 650 u . This limiting parameter would normally prohibit the ITMS from

observing most polypeptide and protein ions if the quadrupole ion trap mass spectrometer could not be operated in such a manner as to extend its nominal m/z range.^{8,108} By rearranging Equation 1-3 as follows:

$$\frac{m}{z} = \frac{8V}{q_z(r_0^2 + 2z_0^2)\Omega^2} \quad (2-1)$$

where $z = ne$ (the integral number of charges [n] times the elemental charge on an ion [e]), one can infer that the maximum m/z range of the QITMS can be increased by decreasing the q_z of ejection.⁸ In other words, applying a lower frequency to the endcap electrodes for resonant ejection will increase the observed maximum m/z of ions in the resultant mass spectrum. Figure 2-2 is a μ ESI mass spectrum of myoglobin displaying the ability to extend the m/z range on the ITMS as the $q_{z,eject}$ was decreased to 0.303, providing a maximum m/z of 1950.

While mass range extension on the ITMS is advantageous for analyzing high m/z ions,^{8,119} there are a number of drawbacks to operating the ITMS in this mode. As a consequence of extending the m/z range, the effective scan rate increases and the degree of precision control of the main RF voltage decreases. Although the absolute rate at which the main RF amplitude is ramped remains unchanged when the m/z range is extended, the rate of change in ion frequency as the RF voltage is increased is greater at lower q_z values since the difference in the ions' frequencies is smaller. In simpler terms, the m/z range that the main RF sweeps per unit time is equivalent to the factor of mass range extension. The result of the increased scanning rate on the QIT is a concomitant decrease in the mass resolution.^{7,120} This is revealed by comparing the peak widths of the +15 charge state ion of myoglobin (m/z 1131) from the μ ESI-MS data on the ITMS

(Figure 2-2) and the LCQ (data not shown). At 50% of the peak height, the $(M+15H)^{+15}$ from myoglobin has a width of 2.31 u on the ITMS, while on the LCQ, which scans at the “normal” rate, the peak width is 0.61 u . This loss of mass resolution would have increased the relative error in the measurement of the uptake of deuterons in H/D exchange reactions with the protein ions.

The other problematic effect of extending the m/z range via decreasing the $q_{z,eject}$ is the loss in the number of digital-to-analog converter (DAC) steps per mass-to-charge associated with controlling the main RF amplitude. The ITMS, being a product of mid-1980s technology, has a 12-bit DAC controlling the main RF voltage, which is equivalent to 4096 discrete levels or steps to set the amplitude of the main RF waveform on the ring electrode. When divided over the nominal mass range of the ITMS (650 u), there are 6.3 DAC steps per unit m/z for the main RF voltage to be set at. In other words, at a fixed RF amplitude an ion at a given m/z will be stored in the QIT within 0.16 u from its closest neighbor $((6.3 \text{ DAC}/u)^{-1})$. However, as the mass range is extended, those 4096 DAC steps are distributed over a larger m/z range. Hence, when the $q_{z,eject}$ is lowered by a factor of 3 (as in Figure 2-2), the number of DAC/ u is reduced to 2.1. This means that the precision to which an ion can be controlled by the ramping of the main RF is $(2.1 \text{ DAC}/u)^{-1} = 0.48 \text{ } u$.

To further illustrate the basis of this problem, a close comparison of the results from H/D exchange reaction of the $(M+17H)^{+17}$ ion from myoglobin is warranted. Figure 2-3 displays the +17 charge state ions produced from μ ESI, isolated in the QIT and allowed to react with D_2O for periods of 0 and 1 seconds. It is obvious from these data that the myoglobin ions that were allowed to react with D_2O have shifted to a higher m/z .

What is uncertain is whether that mass shift is due to the uptake of deuterons or the adductation of D_2O molecules to the $(M+17H)^{+17}$ myoglobin ions. Furthermore, the observation of higher m/z peaks on the $t = 1$ s peak may arise from additional D_2O adduct molecules, a difference in the number of exchanged deuterons due to different ion conformations, or from ion shifting attributed to instability in the main RF DAC. Since the mass range for these data was extended by a factor of 3, a shift in the ion position by one step of the main RF DAC will result in a mass difference of $0.48\ u$. However, this problem is amplified by the fact that the myoglobin ions are multiply charged. For the $+17$ charge state ion, an m/z shift of $0.48\ u$ equates to an absolute mass difference of $8.2\ u$. On the ITMS, it was not uncommon to observe short-term (i.e., scan to scan) shifts in an ion's m/z equivalent to ± 2 DAC steps. For long acquisitions (e.g., hours), the net shift in an ion's m/z assignment could have been even greater due to instability in the main RF DAC. Therefore, the precision and accuracy of the H/D exchange data acquired over the period of minutes and hours would be highly questionable since the data obtained with a reaction time of 1 second were equivocal.

A possible solution to these problems is to reduce the main RF scan rate with the use of a secondary control DAC. This is accomplished by attenuating the scale of the main RF DAC and adding the secondary DAC to provide the DC offset to adjust the main RF voltage to the desired level for the m/z range to be analyzed.¹²¹ The necessary modifications to the ITMS circuitry have been implemented by the Yost group¹²² and others^{7,8,120,121} to be able to scan the main RF at a reduced rate. The advantages of using this methodology is improved mass resolution and the restoration of the DAC steps controlling the main RF "lost" when the mass range is extended. However, the access to

those main RF DAC steps requires a concomitant reduction of the mass range that can be scanned. Also, slowing the scan rate of the main RF causes a decrease in detection sensitivity.^{8,123} Attempts at acquiring μ ESI-MS data on the ITMS were unsuccessful in part due to the computer interfacing problems with the secondary DAC circuitry. The difficulty in tracing down these electronics problems with limited information did not offset the marginal gains in mass resolution and mass precision.

The inability to obtain adequate mass resolution and mass precision on the ITMS were the primary factors for abandoning the H/D exchange project. However, there were other instrumental limitations that contributed to the decision to refit the ITMS. On modern QITMS instruments (e.g. the LCQ) the resonant ejection amplitude is linearly ramped along with the main RF amplitude during ion ejection, while on the ITMS a fixed amplitude is used. For a given ion m/z , the peak width is minimized at a certain resonant ejection amplitude based on the main RF scan rate and the pressure in the ion trap. That optimum amplitude increases with increasing m/z of an ion.¹²⁴ If the resonant ejection amplitude is too low, the ion will not be resonantly ejected (until it reaches a $q_z = 0.908$). Conversely, when the resonant ejection amplitude is too great, the ion's peak width increases, possibly due to higher-order field effects near the endcap holes of the QIT.¹²⁵ Another major reason for developing the new QITMS is that MS/MS experiments on the ITMS were difficult. Specifically, isolation of ions was accomplished via a method called "forward/reverse scanning"¹²⁶ in which low m/z ions were first ejected at $q_z = 0.908$ (forward) followed by ejecting high m/z ions by resonant ejection at a low q_z as the main RF voltage was ramped down (reverse). This method of ion isolation was imprecise in that the efficiency of high m/z ion ejection depended greatly on the q_z of

ejecting the high mass ions and the amplitude of the resonant ejection waveform: too low an amplitude and the high m/z ions would remain; too high and the ion of interest would be ejected from the ion trap. Accomplishing the optimal parameters for ion isolation by forward/reverse scanning on the chromatographic time scale for different m/z ions would have been difficult at best.

Construction of the New QITMS

The aim for the development of this new QITMS (hereafter termed PHOENIX) was to create a mass spectrometer analogous to the commercially available ThermoFinnigan LCQ, but with the ability to modify the operating parameters of the PHOENIX instrument for maximum experimental flexibility. ThermoFinnigan graciously donated to the University of Florida a complete GCQ instrument, which included the ion trap electrodes, control electronics and main RF coil. Additionally, an agreement with the ThermoFinnigan Corporation permitted use of the advanced instrument control software and GCQ source code. Access to the instrument code was vital to adapting the GCQ hardware to the operation of the redesigned QITMS described below.

The initial step to adapting the GCQ components to the existing ESI-ITMS instrument¹¹³ was to replace the ITMS ring and endcap electrodes with the GCQ electrodes. The purpose for changing the ion trap electrodes was to increase the nominal mass range of the instrument as a result of the reduced dimensions of the GCQ electrodes (see the section The Main RF Coil below). Since all the other internal components of the ESI-ITMS (e.g., octopole, electron multiplier) would be retained on the PHOENIX instrument, integration of the GCQ ion trap electrodes into the analyzer assembly without

affecting the relative positions of the existing ITMS elements required careful planning. Therefore, a precise mechanical drawing of the existing ITMS analyzer assembly was prepared using the AutoCAD program (Figure 2-4). This three-dimensional rendering of the ESI-ITMS analyzer assembly made visualization from all angles possible and greatly simplified the arranging of the GCQ ion trap electrodes within the assembly.

Figure 2-5 displays the PHOENIX analyzer assembly containing the GCQ ion trap. With the assistance of AutoCAD, the positioning and modifications of the GCQ ion trap were meticulously calculated before the electrodes were physically altered. Careful attention to detail was necessary to prevent any mistakes in the design to the PHOENIX analyzer since there were no spare ion trap electrodes to correct any design flaws. Some of the considerations in the analyzer assembly design included the lateral positions of the octopole and analyzer tube lens, such that these ion optics would not be in contact with the entrance and exit endcaps, respectively, and the rotational arrangement of the endcap electrodes. The later of these considerations is better illustrated by examining Figures 2-5 and 2-6. The analyzer assembly is held in place between two anodized aluminum plates by three locking pins arrayed at 120° (Figure 2-5). The ITMS trap electrodes have diameters smaller than the inscribed diameter of those locking pins (Figure 2-4) and therefore the ITMS ion trap does not impede the locking pins. However, the diameters of the GCQ endcap electrodes are significantly larger than the ITMS electrodes and it became necessary to section out part of the GCQ endcap electrodes to allow the locking pins to be unobstructed by the endcaps as indicated in Figure 2-6. Additionally, three through holes in the GCQ endcap electrodes were required to permit ceramic spacers to be secured between the endcaps and their adjacent analyzer element. It should be noted

that these modifications in no way affected the performance of the QIT since the hyperbolic portions of the GCQ endcap electrodes were unaltered. Finally, the previously existing mounting holes in the GCQ endcaps had to be oriented to align with one of the through holes in the anodized locking plates to permit the electrical connections to the various electrodes to pass unobstructed. Since only one of the GCQ endcap holes would completely overlap with the existing locking plate through holes, the rotational position of the endcaps was ultimately dictated by the helium gas inlet connector. The dimensional constraints of the vacuum manifold prevent attaching the helium line to the endcap connector before inserting the analyzer assembly into the vacuum chamber. Therefore, the endcaps were oriented such that the gas connectors face toward the top of the vacuum manifold, where an o-ring sealed opening exists, to facilitate the manual connection of the helium inlet line.

The Main RF Coil

On the GCQ instrument, the main RF coil is contained within the body of the GCQ vacuum manifold. To prevent the destruction of that manifold, a new shielded enclosure was constructed to house the GCQ main RF coil. The design of the RF coil box was based on the ITMS shielded container. Six ¼" thick aluminum plates are bolted together to form the RF coil box as illustrated by the three-dimensional wireframe drawing in Figure 2-7. The new main RF coil box provides efficient shielding from environmental electrical noise by mounting directly onto the high voltage feedthrough conflat of the PHOENIX vacuum manifold. Also, the additional surface area of the coil box provided the necessary space to mount the DC Offset (ring electrode), RF Control, and Analyzer printed circuit boards (PCBs).

As mentioned above, one of the primary reasons for switching from the ITMS to the GCQ platform is to extend the nominal m/z range of the instrument. The m/z range of the GCQ is higher than that of the ITMS due to its higher maximum AC voltage applied to the ring electrode (8,500 vs. 7,500 V_{0-p}) and due to the reduced electrode dimensions of the GCQ, where r_0 and z_0 are 7.07 mm and 7.83 mm, respectively, for the GCQ ion trap as compared to 10.0 mm (r_0) and 7.85 mm (z_0) on the ITMS. Even so, the maximum m/z range (1000 u) on the GCQ is not sufficient for many applications using ESI-MS. The mass range can be extended by lowering the resonant ejection q_z in an analogous fashion to that employed on the ITMS; however, some of the drawbacks encountered on the ITMS would also apply to the adapted GCQ. Hence, the best alternative to increasing the nominal m/z range is to decrease the main RF drive frequency (Ω) as indicated by Equation 2-1. Due to the square relationship between Ω and m/z range, a small change in the drive frequency results in a large change in the m/z range of the QITMS.

Briefly, the main RF circuit shown in Figure 2-8 is an LC circuit configured as a bandpass filter¹²⁷ that has a fundamental resonant frequency, ω_0 , given by the equation

$$\omega_0 = \frac{1}{\sqrt{LC}} \quad (2-2)$$

where L is the inductance and C is the circuit capacitance. The main RF coil is a transformer, two electrically isolated coiled wires that inductively couple AC voltage (current) through the surrounding air. Increasing the amount of wire coiled around the non-conductive frame (i.e., coil tank) increases the inductance of the circuit. As Equation

2-2 indicates, a greater inductance on the RF coil decreases the resonant frequency of that circuit, which in this case is the main RF drive frequency.

To determine the new resonant frequency of the RF circuit after increasing the number of turns on the coil, the standing wave ratio (SWR) bridge method was employed. This method, documented by William Fies at ThermoFinnigan in the mid-1980s,¹²⁸ uses a Wheatstone bridge¹²⁷ (Figure 2-9) inserted in series between a frequency generator and the load (i.e., the main RF coil (Figure 2-8)). The bridge serves to detect and nullify the amount of reflected AC voltage from the LC circuit. Details on how this device is constructed and operates are given elsewhere^{113,128} and only a simple overview is described here.

The purpose of the SWR bridge method is two-fold. First, the SWR bridge helps the user to tune the RF coil circuit to its resonant frequency, ω_0 . This is accomplished by measuring the bandpass frequency for the LC circuit (see below). Second, the SWR bridge permits to operator to determine the impedance of the RF coil circuit. Since the RF amplifier is engineered to drive a 50 ohm load, the impedance on the output side of the SWR bridge in Figure 2-8 must be “matched” to 50 ohms to minimize the power required to drive the RF circuit by the RF amplifier. When this condition is satisfied (i.e., $Z_{\text{input}} = Z_{\text{output}} = 50 \text{ ohms}$), no power is reflected back from the load to the RF amplifier and the circuit has a standing wave ratio of 1:1.

The signal measured by the SWR bridge can be monitored via an oscilloscope to yield data as shown in Figure 2-10. Prior to testing the RF coil, the scope display was adjusted such that the time axis (x-axis) correlated to the full range of the frequency sweep and that the voltage scale (y-axis) yielded a signal deflection to the first and third

graduals (from the bottom) using 50 and 100 ohm loads, respectively, on the SWR bridge. As the frequency of the AC waveform is swept, the signal “dips” when the resonant frequency of RF coil circuit is attained. For the modified RF coil on PHOENIX, the fundamental resonant frequency in cycles per second ($f_0 = \omega_0/2\pi$) is 779 kHz. The impedance for the RF coil circuit was matched to approximately 50 ohms by changing the GCQ matching capacitor, C_m in Figure 2-8, from 1500 pF to 5000 pF. The matched output impedance was verified by the SWR signal in Figure 2-10B. The level of the resonant “dip” correlates to 55 ohms, which equates to a standing wave ratio of 1.1:1.0 (i.e., $55 \Omega/50 \Omega = 1.1$ to 1.0). According to the Fies report,¹²⁸ an SWR better than or equal to 1.2:1.0 is an indication of a properly tuned circuit.

A final problem that had to be addressed concerns the RF amplitude modulation portion of the RF coil circuit. Due to the linear relationship between an ion’s m/z and the amplitude of the AC waveform applied to the ring electrode (Equation 2-1), the RF voltage must be precisely monitored to obtain accurate mass spectrometric data. The RF Control PCB of the GCQ and LCQ instruments measures and modulates the RF amplitude applied to the ring electrode. The circuits on both instruments are equivalent with one important exception, the detect capacitor. This air gap capacitor is connected to the RF coil in parallel with the ring electrode and samples a fraction of the main RF voltage for transmission to the RF Control PCB.

When the main RF modulation diagnostics were initially run on the PHOENIX instrument it was observed that the full AC amplitude of 8,500 V_{0-p} could not be achieved. To troubleshoot this problem, the RF shield box was opened slightly during the diagnostic. *(Extreme caution should be taken when the RF shield is opened while the*

instrument is scanning). Upon inspection, it was discovered that the high voltage was arcing in the vicinity of the detect capacitor. Originally it was hypothesized that an exposed sharpened metal site on the GCQ detect capacitor was the cause of the arcing; however, careful cleaning of the capacitor plates and elimination of any possible sharp conducting areas did not alleviate the arcing problem. After discussions with Dr. Scott Quarmby at ThermoFinnigan, installation of an LCQ detect capacitor into the GCQ electronics on the PHOENIX instrument eliminated the high voltage arcing.

The reasons for the arcing problem and why they were corrected by changing the detect capacitor can be rationalized by examining the following three equations

$$X_c = \frac{1}{C2\pi f} \quad (2-3)$$

$$Q = CV_c \quad (2-4)$$

$$C = \frac{\epsilon_0 K_d A}{d} \quad (2-5)$$

where X_c is capacitive reactance, f is the frequency of the AC voltage, C is capacitance, Q is the quantity of charge, V_c is the voltage across the capacitor, ϵ_0 is the permittivity constant, K_d is the dielectric constant of the material between the capacitor plates, A is the area of the capacitor plates and d is the distance between the capacitor plates. When the number of turns on the main RF coil was increased, the frequency applied to the ring electrode and detect capacitor was decreased. Also, the additional turns on the RF coil increased the output voltage from the secondary side of the RF coil to the ring and detect capacitor, since the number of turns on the primary of the RF coil was not altered. As indicated in Equation 2-3, the decrease in the AC frequency passing to the detect

capacitor causes an increase in the capacitive reactance. This increased reactance causes a larger potential drop across the detect capacitor, V_C , that results in a greater charge being retained on the capacitor plates (Equation 2-4). It is theorized that the increased charge capacity of the GCQ detect capacitor in concert with the modified RF coil was the source of the arcing problem.

The LCQ detect capacitor has a larger surface area on the low voltage plate, which, according to Equation 2-5, increases the capacitance relative to the GCQ detect capacitor. This increased capacitance apparently offsets the decreased AC frequency employed on the LCQ and PHOENIX instruments to provide a nearly constant capacitive reactance (Equation 2-3) for all ThermoFinnigan QITMS instruments, which is logical as the RF Control PCB on the GCQ and LCQ instruments are identical. The adjusted capacitive reactance on PHOENIX lowers the voltage across the installed LCQ detect capacitor, thereby decreasing the stored charge on the detect capacitor (Equation 2-4) and eliminating the source of arcing.

Electronics Issues

Since the GCQ was designed as a GC/MS instrument with an electron ionization (EI) and chemical ionization (CI) source, a number of the GCQ circuit elements had to be rewired to function with an ESI source and the ion optics on PHOENIX. Starting at the ion source and moving back toward the detector, each of the PHOENIX components under direct control of the GCQ electronics are discussed below.

The Analytica ESI source used on PHOENIX was previously modified to include a heated capillary inlet^{111,113} to introduce ions into the high vacuum region of the mass spectrometer and to assist droplet desolvation during the ionization process. This heated

capillary is a ThermoFinnigan design and uses a 15 ohm, 24 volt AC ceramic heating element within the stainless steel body of the capillary. Additionally, a 100 ohm platinum resistive temperature detector (RTD) mounted inside the heated capillary assembly serves to monitor its temperature electrically. The GCQ electronics contains circuitry to monitor RTDs for its two heating elements: the ion source and GC transfer line cartridge heaters. The GCQ ion source heating circuit is designed to drive three 7 ohm, 24 volt AC cartridge heaters in series, or in other words, a 21 ohm load. Therefore, the heated capillary, having a lower resistance, would provide a greater load on the GCQ heating circuit. Fortunately, this would not present a problem given that the GCQ circuit contains a 2.5 amp fuse in series with the load, thus preventing excessive current to be drawn by the heated capillary. The GCQ ion source heaters and RTD were successfully wired via the GCQ Analyzer PCB to the body of the heated capillary. The heated capillary temperature is set at the computer terminal by accessing the Ion Source Temperature on the GCQ Tune window.

To guide ions from the ion source to the QIT on the GCQ, an Einzel lens comprised of three electrodes is employed. Three independent voltages are applied to these electrodes, including one programmed to pulse ions to the ion trap during the ion injection segment of the QITMS scan function. The DC voltages to these lenses, termed Lens 1, Gate and Lens 3 on the GCQ Analyzer PCB, were wired to be applied to the heated capillary, ESI tube lens and analyzer tube lens, respectively. Hence, on the PHOENIX instrument, the ESI tube lens serves as the primary element for gating ions to the QIT.

It should also be noted that in addition to the hardware adjustments to these circuits, software changes were required to properly transmit the ESI generated ions on PHOENIX. In the positive ion mode, the GCQ uses negative voltages on Lens 1, Gate and Lens 3 to create a potential well for the positive ions to move toward the QIT. However, on an ESI-MS instrument, progressively decreasing positive voltages are applied to the ESI source optics to create the negative potential well for guiding positive ions to the mass spectrometer. For the PHOENIX analyzer tube lens, a positive voltage is required in the positive ion mode to prevent the large negative electric field created by the conversion dynode from penetrating into the QIT (see Figure 2-5). Therefore, the polarity of the GCQ lens voltages need to be switched from their “normal” programmed settings. Since the GCQ was originally designed to operate in both the positive and negative ion modes, the range of voltages for Lens 1, Gate and Lens 3 covered both positive and negative DC potentials, and the GCQ electronics simply required a command to set the polarity of that voltage. The change in the lenses’ polarities was effected by accessing the GCQ source code software, which is written in the C++ programming language. By carefully reading through the code, it was determined that the polarity of these lenses were controlled in the file “tsave.cpp”. A simple modification of the code altered the polarity settings for these lenses and provided the proper voltages to be applied to the heated capillary, ESI tube lens and analyzer tube lens. Many other changes to the GCQ source code such as the one described here were ultimately necessary to obtain ESI-MS data on PHOENIX, but these changes are not detailed unless required within the context of the discussion.

The initial μ ESI-MS data acquired on PHOENIX presented in this chapter were obtained using the unaltered octopole circuitry developed by Stephenson,¹¹³ which was not under control of the GCQ electronics. Operation of the PHOENIX instrument with this configuration did not pose a problem to acquisition of these data. However, during the initial testing and optimization of the instrument it was discovered that the gating ability of the ESI tube lens was inadequate, particularly for high mass (not high mass-to-charge) ions (see below). To alleviate this problem the octopole circuitry was revised such that it was under direct control of the GCQ electronics, even though the GCQ instrument does not have an octopole. The implications of transmitting high mass ions and the adaptations made to the octopole circuitry are discussed further in the section Gating Ions to the QIT.

The electron multiplier used on PHOENIX is the same one used previously for the ESI-ITMS instrument. It was hoped that simply connecting the multiplier anode to the GCQ's electrometer circuit with RG58 coax cable would have been sufficient to acquire μ ESI-MS data. Unfortunately, this did not prove to be the case; when the multiplier voltage was applied, the electrometer signal was "pegged" off-scale even though no ions were being generated (i.e., the ESI source was not in use) as indicated in Figure 2-11. Initially, it was hypothesized that a ground loop arising from grounding both terminal points of the coax shield may have been causing this "signal"; however, disconnecting either end of the coax shield (to the multiplier anode or to the electrometer) and disconnecting both termini of the shield had no effect.

Since the electrical feedthroughs on the PHOENIX vacuum manifold were coax connectors and coax cable apparently could not be used with the GCQ electrometer, an

alternative had to be found. The current generated by an electron multiplier, even with a gain of 10^6 , is low, typically on the order of 10^{-10} amps or less. Therefore, a shielded transmission cable is necessary to inhibit the pick-up of environmental electrical noise. It was decided to test a twisted pair cable in which the pair of insulated transmission wires was connected together. (Author's note: this is not the proper use of twisted pair cable as one insulated wire is normally used to transmit the signal and the other is used to transmit either a return signal to the source or noise to a ground source). Figure 2-12 displays the effects of the twisted pair cable to the electrometer signal. When the uninsulated shield wire is left unconnected to a ground point adjacent to the multiplier anode and electrometer inputs, a periodic signal is observed, which was still very high but no longer off-scale (Figure 2-10A). A similar signal is seen if the twisted pair shield is grounded only near the multiplier anode input (data not shown). The signal drops to near zero (Figure 2-10B), however, upon connecting the shield terminus to ground only at the input of the electrometer. This is the configuration adapted for the PHOENIX by placing RG58 terminal connectors on the twisted pair cable and by grounding the shield on the electrometer input. It is uncertain why the twisted pair cable works and the coax cable does not. One possible explanation is that noise picked up by the coax cable shield is capacitively coupled through the dielectric material to the center conductor causing a portion of the noise to be transmitted to the electrometer.

Much like the octopole circuit, the voltage to the conversion dynode on PHOENIX was initially provided by a power supply independent of the GCQ. The reasons for this were that the ESI-ITMS instrument used this setup without problem and the independent power supply provided a variable 0-20 kV setting whereas the GCQ

dynode power supply output a fixed 15 kV. The data acquired on the ESI-ITMS instrument used a dynode voltage of -10 kV (for positive ion analyses) and it was uncertain what effect the higher potential would have on the data acquired on the PHOENIX instrument. To determine if the GCQ dynode power supply could be used on the new instrument, the effect of the conversion dynode voltage on the $(M+H)^+$ ion intensity of neurotensin fragment 1-6 (m/z 777) was measured. Figure 2-13 shows that the ion signal increases rapidly at dynode voltages between -2 kV and -5 kV then increases more slowly until it plateaus at approximately -12 kV, where it remains steady up to and including -15 kV. Hence, the GCQ dynode power supply was coupled to the PHOENIX conversion dynode since the ion signal is independent of the dynode voltage above -12 kV. Interestingly, when the dynode voltage was set at -1 kV, the ion signal nearly disappears. This can be attributed to the fact that the electric fields created by the dynode and the electron multiplier (set at -1400 V) are nearly equivalent and offset each other. The result is that the ions ejected from the QIT pass by the off-axis dynode and multiplier, and therefore are undetected.

Optimization of the QITMS

A series of standard solutions with varying molecular weights was infused to the μ ESI source to calibrate the newly developed PHOENIX quadrupole ion trap mass spectrometer. An overview of the processes and experiments required to optimize the numerous parameters affecting the operation of the QITMS is presented in this section.

Mass Calibration

The first and foremost parameter to calibrate on any mass spectrometer is the assignment of ions' mass-to-charge. On the QITMS there are two primary factors that

affect the mass calibration of ions at a constant scan rate and fixed q_z , eject: the helium buffer gas pressure and resonant ejection amplitude. To complicate the mass calibration process, the optimal resonant ejection amplitude varies with m/z and is dependent on the pressure of the helium in the QIT.

One of the purposes for the helium bath gas is to remove excess kinetic energy from the externally injected ions within the QIT so that they will maintain “infinitely” stable trajectories.³⁸ Typical helium pressures employed in analytical QITMS are on the order of 1-2 mtorr. Higher helium pressures improve the trapping efficiency of injected ions, although this advantage is offset by a sacrifice in mass resolution¹²³ and MS/MS efficiency.¹²⁹ Since a pressure gauge was not connected directly to the PHOENIX ion trap, the helium pressure was monitored indirectly by the ion gauge pressure in the third differentially pumped region. Figure 2-14 displays the effect of helium pressure on ion intensity. The injection efficiency for low m/z ions ($<1000\ u$) tends to level off at helium pressures corresponding to 9.0×10^{-6} torr on the ion gauge, whereas higher m/z ions ($>1000\ u$) seem to optimize at somewhat higher helium pressures. This can be attributed to the fact that high m/z ions require a larger number of collisions with the helium buffer gas to remove a sufficient amount of kinetic energy to remain stable within the QIT.^{43,44}

Optimizing the QIT helium pressure in this manner was initially satisfactory. However, the pressure reading on the ion gauge does not remain constant over long periods of time. The differences in the ion gauge pressure may or may not be due to changes in the helium pressure. Therefore, the method for setting the helium pressure in the QIT was later altered to monitoring the MS/MS data for a well-characterized compound. Spiiperone has been thoroughly studied in the Yost group on many different

QITMS instruments.¹³⁰⁻¹³² Based on those previous investigations and the author's experiments with spiperone on the LCQ, it was determined that the helium pressure could be adjusted until the maximum MS/MS efficiency was realized for the spiperone $(M+H)^+$ by using a resonant excitation amplitude of $0.90 V_{pp}$ at $q_z, excite = 0.225$ for a period of 15 ms. When the helium pressure is too low, the intensity of the spiperone product ions (e.g., m/z 291, 262, 165) is greatly reduced. Conversely, if the helium pressure in the QIT is set too high, the above product ions are not observed. This phenomenon is caused by the inability of the resonantly excited ions to gain sufficient kinetic energy to undergo dissociative collisions due to the additional collisional cooling capacity at elevated helium pressures.¹²⁹ Adjusting the helium bath gas pressure using this method yields the best results when the spiperone product ions are maximized and the precursor $(M+H)^+$ ion is reduced to $\leq 5\%$ relative abundance of the product ions.

In contrast to the ITMS, the resonant ejection amplitude on the newer ThermoFinnigan QITMS instruments is ramped during ion ejection. Accordingly, the amplitude for the resonant ejection waveform requires calibration as a function of m/z . Initial resonant ejection amplitude experiments required manual data processing to ascertain the optimal voltages for the calibrant ions, which were highly time consuming. To overcome this time sink, a Visual Basic routine was written to simultaneously monitor the peak widths of several ions as a function of the resonant ejection amplitude. An example of the results collected from this program is given in Figure 2-15 for the $(M+H)^+$ of spiperone. At low resonant ejection amplitudes, the peak width is quite wide due to the fact that the ions are not being resonantly ejected in an efficient manner at the resonant ejection q_z of 0.902, but rather are ejected at the $\beta_z = 1$ boundary ($q_z = 0.908$).

Although it is difficult from Figure 2-15 to accurately identify a single resonant ejection amplitude corresponding to the minimum peak width, it is advantageous to select the minimum peak width at the lowest resonant ejection amplitude. Upon visual inspection of Figure 2-15, that local minimum resonant ejection amplitude is at approximately 4.0 V_{pp} . Due to the deleterious effect of the increased peak widths at higher resonant ejection amplitudes, which has been investigated recently (*vide infra*),¹²⁵ the minima in a series of these peak width-voltage curves are fit to a line as a function of m/z (Figure 2-16). This equation is input to the "INST.ini" file in the GCQ control software to provide the m/z -normalized resonant ejection amplitude for mass analysis.

Once the helium buffer gas pressure and resonant ejection amplitude are optimized, the QITMS is mass calibrated by adjusting the slope and intercept of the digital-to-analog converter (DAC) circuit controlling the main RF amplitude ramp. Altering the main RF DAC parameters is accomplished via the variables *mslope* and *mintercept* in the "INST.ini" file. Infusing several compounds of known mass and monitoring the resultant μ ESI-MS, the *mslope* is adjusted until the m/z of all the observed ions are either greater than or less than their calculated m/z . Once this condition is satisfied, the *mintercept* is changed until all the ions' m/z correspond to their correct m/z to within $\pm 0.1 u$. This process requires manual adjustment of the "INST.ini" file and may take several iterations to properly align the m/z of the ions; however, once mass calibration is realized, the m/z assignment of the ions is stable to $\pm 0.2 u$ for weeks or even months at a time. Furthermore, a Visual Basic program could be devised to make the mass calibration process semi-automatic, but in the interest of time this option was not accomplished.

Mass Range of PHOENIX

One of the main goals of developing the new QITMS was to create an instrument with a sufficient m/z range to examine analytes produced by ESI without sacrificing instrument performance. By incorporating GCQ electronics and ion trap electrodes and by decreasing the drive frequency of the main RF voltage, a greater nominal m/z range was attained. Figure 2-17 demonstrates the m/z range of PHOENIX. High concentration inorganic salt solutions produce cluster ions by ESI¹³³ and serve as an ideal high m/z calibrant, especially when the atoms of the salt are monoisotopic as is the case for sodium iodide. The highest positive cluster ion observed on PHOENIX was $(\text{NaI})_{11}\text{Na}^+$ at m/z 1671.6 (Figure 2-17A). To better validate the predicted maximum m/z of 1800 on PHOENIX, the polypeptide Sendai Virus Nucleoprotein Fragment 321-336 with a monoisotopic mass of 1779.8 u was examined by μ ESI-MS. As seen in Figure 2-17B, the $(\text{M}+\text{H})^+$ ion is observed at m/z 1780.9, clearly demonstrating the mass range of 1800 u on PHOENIX.

The extended m/z range of PHOENIX helped to overcome one of the major shortcomings of the ITMS instrument, yet it did not eliminate all the problems. Specifically, the GCQ data system has a limited number of sampling points for data acquisition, and increasing the m/z range caused a concomitant decrease in the number of data points per u . For example, when the GCQ scans an m/z range of 1000 u , the data system acquires approximately 15 data points per u . However, when scanning a range of 1500 u on PHOENIX, only about 10 samples per u can be acquired, otherwise the data system crashes. The result of this data limitation when monitoring a wide m/z range is a reduction in the mass resolution by undersampling an ion peak. Nevertheless, the

reduced data sampling on PHOENIX is still superior to the number of data points per u on the ITMS, particularly compared to the ITMS when the m/z range was extended by lowering the resonant ejection q_z .

Ion Transmission and Ion Injection

Since the PHOENIX instrument uses an ion source external to the QIT, ions must be transported from the ESI source to the ion trap for storage prior to acquisition of a mass spectrum. Ion transmission is affected mainly by electric potentials between the source and QIT. Varying the voltages on the ion optic elements serves to efficiently transport ion to (or deflect ions from) the QIT. However, the optimal voltage applied to a given lens is often coupled to the potentials of adjacent lenses, which can complicate the tuning process. Fortunately, previous work on the ESI-ITMS and the LCQ instruments provided guidelines on the potential ranges that could be used as reference voltages for tuning the PHOENIX lenses.

The Visual Basic (VB) custom tune software controlling the GCQ electronics had already been programmed to allow dynamic tuning of the lenses. As discussed in the Construction of the New QITMS section above, the heated capillary offset (lens 1), ESI tube lens (gate) and analyzer tube lens (lens 3) were under direct control of the VB software and were tuned using the existing lens tuning routine. An example of the results for tuning the heated capillary DC offset is provided in Figure 2-18. At lower voltages on the heated capillary, the ion signals are relatively constant. As the heated capillary offset is increased above 50 V, two trends are observed: the ion signal for the m/z 396 ion decreases and the intensities for the m/z 291 and 777 ions increase. These trends can be rationalized by examining the experimental conditions experienced by these ions. The

heated capillary transports ions from atmosphere to the intermediate vacuum region (approximately 1 torr). As the ions exit the heated capillary they experience free jet expansion^{97,98} as discussed in Chapter 1. When the potential difference between the heated capillary and the skimmer cone is sufficiently high, the ions can be accelerated beyond the velocity dictated by the pressure drop. This increased velocity causes the ions to undergo collisions of higher energy with the surrounding gas molecules. For low m/z ions, this may induce dissociation of the ions, as is the case for the $(M+H)^+$ of spiperone at m/z 396 producing the m/z 291 fragment ion. For ions of higher m/z , collision-induced dissociation (CID) is not favored at the kinetic energies used in the experiments displayed in Figure 2-18. This is due to the large differences in the masses between these high m/z ions and the surrounding neutral gas molecules (mainly N_2). As Equation 2-6 indicates, the maximum amount of energy that can be imparted to the ion (E_{REL}) is a small percentage of the ion's kinetic energy (E_{LAB}) based on the large differences in mass between the neutral (N) and the ion (m_p).³

$$E_{REL} = \left(\frac{N}{N + m_p} \right) E_{LAB} \quad (2-6)$$

Furthermore, any energy absorbed by the higher m/z ions can be dispersed over the increased number of vibrational and rotational degrees of freedom of these larger ions, thereby inhibiting bond dissociation. This process of vibrational and rotational excitation, however, will cause ion desolvation and declustering processes to be favored, hence leading to improved sensitivity for high m/z ions at greater heated capillary offset potentials (Figure 2-18).

Optimization of all the ion optical elements was conducted in a similar manner; the results of those experiments are listed in Table 2-1. The optimal voltages for some of the lenses (e.g., heated capillary) are a compromise between the best-observed signal for certain ions and the minimization of ion fragmentation for others. The voltages employed for ion transmission on PHOENIX by no means represent an exhaustive study of optimal ion transmission and ion injection, and other values on these elements may provide better results. For example, the LCQ uses an ion trap offset of -10 V, whereas a DC offset of -7.5 V is employed on PHOENIX. Such an intensive study of ion transmission and ion injection was not the focus of this dissertation.

The injection of ions into the QIT is a function of a number of parameters including an ion's kinetic energy, the amplitude of the main RF and the phase of the main RF.^{43,44} Additionally, the optimal amplitude of the main RF for ion injection changes with m/z . This is demonstrated in Figure 2-19 where the normalized ion intensity for various ions across the m/z range is plotted as a function of low mass cut-off (LMCO). There are two items of interest that can be inferred from Figure 2-19. First, the optimal LMCO, which is directly proportional to the main RF voltage, increases for increasing m/z . This means that a greater RF potential is required to efficiently inject and trap ions of higher m/z . Second, the optimal voltage range of the main RF for trapping ions decreases with increasing m/z . This has the impact of potentially biasing against certain m/z ions based on the amplitude(s) of the main RF during ion injection. The ramifications of this property of the QITMS are significant if one is examining compounds with a wide range of masses, as the case may be during LC/MS analyses.

To better illustrate the effect of the optimal LMCO on the injection of ions into the QIT over a wide m/z range, Figure 2-20 presents these data for a larger set of compounds. Fitting a curve to the optimal LMCO data for the various ions reveals a relationship that appears to have a root power dependence on m/z , even though previous studies demonstrated a linear dependence.⁴³ This discrepancy may be due to the error in the data presented here, as these data were only acquired once, or there may be another undetermined explanation that resolves the differences in these results. Regardless of the reasons for the observed disparity between the two instruments, the overall concept remains the same: the RF amplitude must be varied during ion injection to effectively trap ions with a wide range of m/z .

The LCQ and GCQ instruments trap ions over a wide mass range by employing two or more discrete levels of the RF amplitude during the ion injection period. Two RF amplitude levels are used on the GCQ if scanning over its entire m/z range of 1000 u , while the LCQ, with a mass range to 2000 m/z , employs up to four injection RF amplitude levels. Since the PHOENIX uses GCQ electronics and software, initial data acquired on the new instrument used two RF amplitude levels for ion injection; however, with the extended m/z range of PHOENIX, it was quickly discovered that the lack of additional injection levels imparted a bias against certain m/z ions (data not shown). If LC/MS analyses were to be conducted on PHOENIX, the “gaps” in the injection of various m/z ions would have to be “filled” to examine the entire m/z range of the instrument.

The ion injection discrepancy on PHOENIX was not a hardware limitation, rather it was a shortcoming encoded into the software. Originally, the goal to overcome the ion

injection problem on PHOENIX was to increase the number of RF injection amplitude levels from two to four, as on the LCQ. Access to the GCQ instrument code would permit changes to the ion trap scan function; however, upon close examination of the C++ and VB code, it was determined that extensive editing of the instrument control programs would be necessary to add more ion injection levels to the existing code. An alternative solution was to ramp the main RF amplitude during the ion injection period to provide a more uniform injection of ions over a wide m/z range on PHOENIX. With the existing instrument code, changing from a two-step injection to a linear ramp was a relatively straightforward process. Briefly, the variables setting the first and second RF amplitude levels in the C++ file "MATRIXMS.cpp" were used as the start point and end point for the main RF amplitude, which was recoded to ramp linearly during the time of ion injection rather than holding at each of the RF levels for 50% of the injection time.

A direct comparison of the μ ESI-MS data generated when the main RF was stepped and ramped during ion injection is displayed in Figure 2-21. The compounds analyzed, caffeine, spiperone, neurotensin (1-6), eldoisin and Sendai virus nucleoprotein (321-336), were chosen to yield ions spanning the full m/z range of the PHOENIX instrument. Interestingly, the intensities of the ions are equivalent within experimental error whether the main RF amplitude was stepped (Figure 2-21A) or ramped (Figure 2-21B) during ion injection. This observation can be rationalized by comparing the two injection processes with the injection efficiencies for the ions monitored. When the main RF amplitude is stepped during ion injection, the efficiency of injection for a particular ion may not be optimal but still favorable (Figure 2-19), particularly for low m/z ions, which can be injected into the QIT over a wide range of RF amplitudes. On the other

hand, if the main RF amplitude is ramped during ion injection, the optimal injection RF level for all ions to be analyzed will be attained; yet this optimal injection RF amplitude will only be maintained for a fraction of the total injection time. Therefore, the data in Figure 2-21 may be attributed to a “trapping flux” parameter based on the fraction of time the RF amplitude remains at a certain value and the injection efficiency of a given ion. Whether coincidence or an intrinsic property of the QIT that these particular ions yielded equivalent MS data for the ion injection conditions applied in Figure 2-21, the fact that RF ramping during ion injection will optimally trap all ions in a given m/z range for some fraction of the injection period reduces the potential for negative m/z bias during MS analyses.

Gating Ions to the QIT

Analysis of ions with a QITMS is accomplished using a temporal sequence termed a scan function. In other words, ions are injected into the QIT, stored for a period, manipulated if necessary (e.g., isolated and dissociated for MSⁿ analyses) and ejected for data recording. Therefore, the QITMS works best with ion sources that form pulses of ions, which is not the case with ESI. Since electrospray ionization is a continuous ionization source, ions may pass through the QIT during the mass analysis step, resulting in random chemical noise being observed in the mass spectral data unless some means of deflecting the ion beam from reaching the QIT is employed. The PHOENIX is configured such that the ESI tube lens is controlled by the Gate lens circuit of the GCQ electronics. This allows the application of one voltage to permit ion transmission during the ion injection period (see Table 2-1) and another voltage to inhibit ion transport during the remainder of the scan function. For positive ion analysis, a

negative potential is applied to the ESI tube lens to attract the ion beam off the central axis to collide with the skimmer cone. Hence, the ion gate is "closed" when -130 V, the maximum negative amplitude provided by the GCQ electronics, is applied to the ESI tube lens, which is sufficient for many of the compounds examined on PHOENIX. However, when high mass compounds were examined, the effectiveness of the ESI tube lens to gate these ions was inadequate. Figure 2-22 shows the μ ESI mass spectrum of myoglobin, with a molecular mass (M_r) of $16,950$ u, acquired when the ESI tube lens was set to -130 V for the entire scan function. The individual charge states of the myoglobin ions can be clearly observed along with high chemical background and/or space charge effects of the injection of too many ions into the QIT. Obviously, -130 V on the ESI tube lens is not a large enough negative potential to prevent the positive myoglobin ions from traversing the low-pressure side of the ESI source, through the skimmer orifice to be transmitted to the ion trap via the octopole.

The inability to prevent high mass ions from being transferred from the ESI source to the QITMS is problematic even if high mass analytes are not examined. For example, incomplete solvent evaporation during the electrospray process may lead to the presence of high mass solvent cluster ions that would appear as chemical noise in the resultant ESI mass spectra. Therefore, an additional means of blocking ion transmission to the QIT was required. This was realized by pulsing the AC amplitude on the octopole so that the AC voltage was applied only during the ion injection period of the scan function.

The octopole circuitry used on PHOENIX for the early optimization experiments was the unaltered device originally devised by Stephenson for the ESI-ITMS

instrument.¹¹³ Briefly, a Stanford DS345 waveform generator provided the AC frequency to a TSQ 4500 quadrupole electronics module (QEM), which amplified the AC waveform before transmission to the RF coil and modulated the octopole amplitude supplied from the RF coil to the octopole rods. Since the QEM was independent of the GCQ electronics, the octopole was not under direct computer control and adapting the two systems to work together under one computer platform interface would have been problematic. Hence, an octopole coil from a TSQ 7000 provided by ThermoFinnigan was coupled to the GCQ electronics and the existing octopole on PHOENIX to provide the necessary interface to allow the GCQ electronics to control the octopole amplitude.

Figure 2-23 shows the basic schematic of the revised octopole circuit on PHOENIX. Notice the similarity to the main RF coil circuit on the QITMS (Figure 2-8), with the essential difference being that the secondary of the octopole RF coil is center tapped, which provides the AC voltage to adjacent pairs of octopole rods 180° out of phase. Outlining the operation of the new octopole circuitry, the GCQ Waveform Dynamic Digital Synthesizer (DDS) card, installed in an ISA slot of the PHOENIX control computer, supplies the AC waveform from the spare “Freq3” channel to a secondary GCQ Waveform Amplifier PCB (the primary GCQ Waveform Amplifier is used for the resonant excitation/ejection waveform to the endcap electrodes). Since PHOENIX uses ESI and not electron ionization, the unused electron energy (EE) DAC from the Main System PCB was available to modulate the output AC voltage from the Waveform Amplifier PCB. RG58 coax cable transmits the amplified signal to the Octopole PCB, where the RF coil further amplifies the AC waveform and applies the voltage to the octopole rods via two RG59 cables. The DC offset to the octopole is

provided to one side of the RF coil secondary from the GCQ electron lens circuit, which normally gates an electron beam into the GCQ electron ionization source. This circuit was modified to reduce the voltage gain of the U17 operational amplifier on the Main System PCB from 13 to 1.3 by changing the inverting input resistor (R109) from 10 k Ω to 100 k Ω while maintaining the feedback resistor (R69) at 133 k Ω . Finally, the octopole circuit was tuned using the SWR bridge method¹²⁸ (see above) to a resonant frequency of 1.667 MHz, to which the *Freq3* variable was set in the VB Custom Tune software.

To obtain an estimate of the AC voltage applied to the octopole rods, one of the output leads from the RF coil secondary was connected to an oscilloscope and the EE voltage, which serves to modulate the octopole AC amplitude, was varied in the VB software. The results of that experiment are plotted in Figure 2-24. Note that the octopole AC amplitude measured by the oscilloscope will not be equivalent to that actually applied to the octopole rods due to the differences in the capacitive loads between the two devices. Above an EE voltage setting of -27 V, the octopole AC amplitude drops to nearly zero. This may be due to the protection circuitry of the Waveform Amplifier PCB, which shuts off once the current draw of the load becomes too great. The Waveform Amplifier was designed to drive a 12 ohm load, whereas the octopole was intended to operate with a 50 ohm amplifier. Therefore, proper impedance matching of this RF circuit could not be readily accomplished.

To ascertain if the limited octopole AC amplitude would cause a problem in the efficient transmission of ions to the QIT, the ion intensity of several ions was monitored as a function of the octopole AC amplitude (as determined by the EE voltage setting). As seen in Figure 2-25, the ion intensity initially increases rapidly at low octopole AC

amplitudes followed by more gradual increases in ion signal until the EE setting reaches about -15 V , corresponding to an octopole AC amplitude of 144 V_{0-p} , where the ion intensities level off or increase weakly with increasing voltage on the octopole. To ensure that these octopole amplitudes were sufficient for high mass ions, myoglobin was examined using the revised octopole circuit. The μESI mass spectrum of myoglobin is given in Figure 2-26, where the octopole amplitude is set to 193 V_{0-p} (EE voltage = -20 V). Clearly, optimal ion transmission is attained well before the “cut-off” AC voltage for the new octopole circuit, even with high mass ions.

The primary goal of revising the octopole circuit was to provide additional gating of the ions generated by the ESI source. The final testing of the octopole circuit set out to confirm that the octopole AC amplitude was properly switching on and off. An oscilloscope measuring the octopole amplitude easily established that the voltage was being pulsed; however, the timing of the AC amplitude modulation was imperative to ensure the ions were being transmitted at the intended time during the scan function.

A mixture of angiotensin I ($M_r = 1295.7\text{ u}$) and neurotensin fragment (1-6) ($M_r = 776.4\text{ u}$) was analyzed by $\mu\text{ESI-MS/MS}$, where the $(M+H)^+$ ion of neurotensin at $m/z\ 777$ was isolated but not dissociated (i.e., not resonantly excited) before mass analysis. Inspection of Figure 2-27A reveals a minor peak at $m/z\ 433$, which from previous analyses is consistent with the $(M+3H)^{+3}$ of angiotensin I and is not a fragment ion from the $(M+H)^+$ of neurotensin (1-6). The ion isolation process on PHOENIX uses resonant ejection via multiple overlapping waveforms with the exclusion of the frequency of the ion of interest, which is held at $q_z = 0.83$. Therefore, all ions, with the exception of the narrow range of ions in the “window” of waveforms, will be eliminated from the QIT.

Taking into account the possibility that the isolation waveforms were not working properly, when the m/z 777 ion from neurotensin (1-6) was moved by the main RF to a $q_z = 0.83$, the LMCO for the ion trap is equivalent to m/z 710, resulting in the ejection on any ion with a lower m/z . Thus, the m/z 433 ion from angiotensin I observed in Figure 2-27A must have been injected *after* the ion isolation step. Note that after ion isolation the main RF moves the m/z 777 ion to a $q_z = 0.225$ (where normally ion dissociation occurs) in MS/MS mode prior to mass analysis. At this lower main RF amplitude, the LMCO is m/z 193 and the $(M+3H)^{+3}$ at m/z 433 from angiotensin I could be injected and trapped in the QIT, provided the octopole amplitude was on. One alternate possibility is that the electrosprayed ions, particularly high mass ions, could traverse the space from the μ ESI source to the QIT when the octopole amplitude is off; however, this was quickly discounted as no signal was observed when the octopole voltage was set to zero for the entire scan function (data not shown). Upon close inspection of the GCQ C++ instrument code (MSNMATRIX.cpp), it was discovered that the EE voltage remains on after the ion isolation step and is not set to zero until just prior to the mass analysis step. When the scan function was altered to turn off the EE DAC voltage immediately after the ion injection step, the m/z 433 ion was eliminated (Figure 2-27B). The C++ code controlling the single-stage MS scan function (MATRIXMS.cpp) was accordingly modified to set the octopole AC amplitude to zero via the EE DAC after the ion injection step.

Optimization in MS/MS Mode

Tandem mass spectrometry is a powerful analytical technique for two reasons: it provides structural information on the analyte ions and it improves the signal-to-noise ratio of analyses.¹³⁴ The unique property of the QITMS is that it can conduct multiple

stages of mass spectrometry (MS^n) with a single mass analyzer.^{6,134} While there are several means of conducting MS/MS experiments such as photoinduced dissociation^{111,113} and surface-induced dissociation,¹³⁵ the most common means is via collision-induced dissociation (CID).^{6,35,136} Increasing the kinetic energy of ions by an acceleration voltage followed by collisions with a neutral target gas converts a portion of that kinetic energy to internal energy, which if sufficient will cause bond dissociation within the ion. On the QIT, the acceleration voltage is termed the resonant excitation amplitude, which is applied to the endcap electrodes as described in detail in the section of the Basic Operating Principles of the Ion Trap in Chapter 1.

Generally, ions of higher m/z require greater collision energies to cause ion dissociation. The primary reason for this is that as an ion's mass increases, it has a greater number of degrees of freedom over which to distribute the extra internalized energy.³ Therefore, the general rule of thumb is that the higher the m/z of an ion, the greater the resonant excitation amplitude required to induced dissociation. However, a caveat with the QITMS is that should the resonant excitation amplitude be too great, the ion may be ejected from the ion trap prior to fragmenting, thereby rendering the MS/MS technique ineffective.

In recent years the problem of applying the proper resonant excitation voltage for obtaining quality MS/MS data on a wide range of analyte ions has been addressed in two ways: m/z normalized collision energy¹³⁷ and multilevel CID.¹²³ Briefly, both methods first calibrate the QITMS in MS/MS mode with a set of standards having precursor ions with a wide variety of m/z to find the ions' optimum resonant excitation voltages. Once these values are obtained, the normalized collision energy method applies the single

optimal amplitude as a function of m/z , while the method of multilevel CID uses a series of voltage values about the optimal resonant excitation amplitude. The latter method has the advantage of obtaining MS/MS data on ions that may be more difficult to fragment than the calibrant ion (ion dissociation is a function of gas-phase structure as well as m/z),¹²³ albeit at longer acquisition times.

Since the PHOENIX instrument was designed to analyze peptide mixtures via capillary LC/MS/MS, a method for acquiring data on a wide range of ions would likely be necessary. Therefore, as described above, the first step is to calibrate the QITMS in MS/MS mode with a series of ions to find the optimum resonant excitation amplitudes. It is important to note that this calibration needs to be conducted whenever the parameters affecting the optimal resonant excitation voltage change, namely the resonant excitation q_z , the resonant excitation time, or the helium pressure in the QIT. Figure 2-28 displays the CID breakdown curves generated from the $(M+H)^+$ precursor ions of caffeine, spiperone and neurotensin (1-6) at m/z 195, 396 and 777, respectively. Two items to note are that the optimal resonant excitation amplitude increases with m/z and that the range of resonant excitation voltages that can be applied for effective ion dissociation also increases with m/z . The reason for the latter is that ions having higher m/z require greater amplitudes to be ejected from the QIT. Hence, this calibration procedure is more critical for low m/z ions.

Owing to its ease of implementation, it was decided that the m/z normalized collision energy method would be incorporated on PHOENIX. The optimal resonant excitation amplitudes for several calibrant ions as a function of m/z are plotted in Figure 2-29. This plot was generated from CID breakdown curves (such as those in Figure 2-28)

using a $q_{z,excite} = 0.225$ and a resonant excitation time of 15 ms. The linear equation from Figure 2-29 was applied in a Visual Basic routine that calculates the resonant excitation amplitude based on the input m/z of the precursor ion to be analyzed by MS/MS. With the added m/z -normalized collision energy option, the need to optimize or estimate the resonant excitation voltage for analytes not previously analyzed by ESI-MS/MS was eliminated.

Sensitivity of the New QITMS

In recent years there have been a number of reports illustrating the ability to detect analytes at low attomole¹³⁸⁻¹⁴² and even high zeptomole¹⁴³⁻¹⁴⁵ amounts using mass spectrometry. With the construction and optimization of the new QITMS with μ ESI interface, the goal was to have a viable instrument that could identify compounds at similar levels. To test the sensitivity of the PHOENIX mass spectrometer, a test peptide, angiotensin I, was examined at varying concentrations in single-stage MS and MS/MS modes.

Before examining the sensitivity limits of the new QITMS, representative μ ESI-MS and MS/MS data of angiotensin I were acquired using a moderate concentration of 1 μ M. Human angiotensin I has the sequence (using the standard one-letter amino acid designation) DRVYIHPFHL, which equates to a monoisotopic mass of 1295.7 u . This decapeptide, having four basic sites, namely the side chains of arginine, two histidines and the amino terminus, takes on multiple protons when subjected to positive ion electrospray ionization as demonstrated in Figure 2-30A. The base peak at m/z 433 in the single-stage MS data is the $(M+3H)^{+3}$ ion for angiotensin I, while the doubly-protonated ion at m/z 649 can also be observed. The μ ESI-MS data in Figure 2-30A were acquired

while infusing a 1 μM solution at a flow rate of 200 nL/min. and represent the consumption of 12 femtomoles of angiotensin I.

If the m/z 433 ion is isolated and subjected to CID, the resultant MS/MS data for the triply protonated angiotensin I ions are observed (Figure 2-30B). Notice that the product ion spectrum for the $(M+3H)^{+3}$ ion of angiotensin I yields peaks at mass-to-charges higher as well as lower than the precursor ion. This is a result of the formation of fragment ions with a lower mass and with a lesser number of charges than the precursor triply charged ion, which results in peaks at higher m/z than the m/z 433 precursor ion. These MS/MS data reveal the peptide sequence of angiotensin I, since peptide ions often dissociate along their peptide bonds.¹⁴⁶ The angiotensin I fragment ions are labeled using the nomenclature proposed by Roepstorff and Fohlman¹⁴⁷ and modified by Biemann,¹⁴⁸ where the y- and b-ions are those in which the charge is retained on the C-terminus and N-terminus of the peptide ion, respectively, and the subscript indicates the number of amino acids from the indicated terminus of that fragment ion.

Starting with an angiotensin I solution of 0.01 nM and increasing the concentration by factors of ten, the PHOENIX instrument sensitivity was tested. The observation of the $(M+3H)^{+3}$ ion of angiotensin I at m/z 433 in single-stage MS mode was realized above the chemical noise at a concentration of 10 nM (Figure 2-31). The $\mu\text{ESI-MS}$ data in Figure 2-31 were acquired using a flow rate of 200 nL/min. over a period of 0.06 min, equating to a sample consumption of 12 attomoles of angiotensin I. Even though the triply charged angiotensin I ion can be discerned amongst the chemical background, the observation of a peak at every m/z makes this identification somewhat

equivocal. Therefore, a more selective method, which may be necessary for unknown analytes, is readily achieved by employing MS/MS.

When the angiotensin I concentration was lowered by ten-fold (i.e., 1 nM) and CID was conducted on the m/z 433 ion, the resultant product ion spectrum demonstrates clear identification of the angiotensin I sequence ions (Figure 2-32). The MS/MS data in Figure 2-32 are an average of three analytical scans requiring 0.15 minutes at a flow rate of 200 nL/min., representing a consumption of 30 attomoles of the angiotensin I peptide. Detection of angiotensin I at a lower concentration (i.e., 1 nM in Figure 2-32 vs. 10 nM in Figure 2-31) using MS/MS results from the elimination of the chemical noise at other m/z and reflects the selective nature of tandem mass spectrometry as provided by the molecular specificity of an ion when it dissociates in the gas phase. If the m/z 433 region is examined in the single-stage MS mode using the 1 nM angiotensin I solution, the $(M+3H)^{+3}$ ion is obscured within the chemical noise (see Figure 2-32 inset).

The utility of tandem mass spectrometry in terms of structural information and improvement in the detection of low-level analyte signals has been demonstrated. However, this testing of the sensitivity of the newly developed QITMS was conducted on a purified standard. A much greater challenge lies in attempting to identifying analytes from samples containing numerous compounds at trace levels, particularly if those samples include high concentrations of buffer salts as is often the case with biological samples. Hence, an even greater degree of selective analysis is required to successfully analyze sample mixtures from a complex matrix. To assist in this challenge, capillary liquid chromatography was adapted to the μ ESI-QITMS system described here.

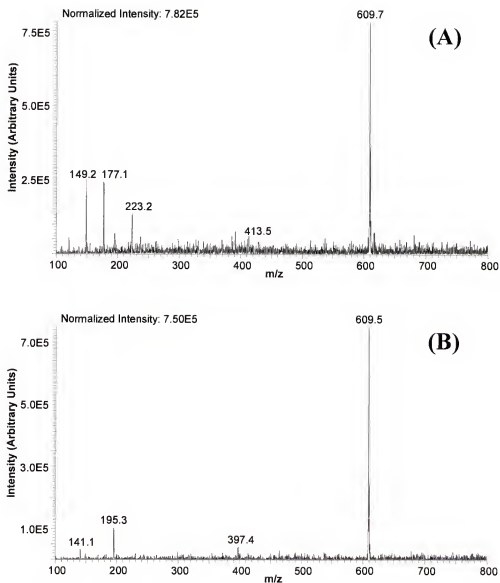


Figure 2-1: ESI-MS data of 100 pg/ μ L reserpine. (A) Mass spectrum using μ ESI source at a flow rate = 0.1 μ L/min. (B) Mass spectrum using the conventional ESI source at a flow rate = 3 μ L/min. The same ionization time was used and an equal number of scans were averaged to yield these data.

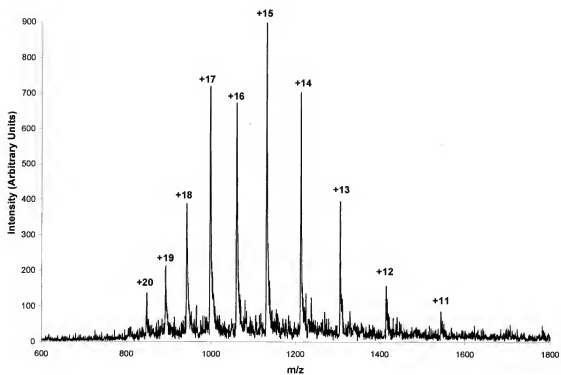


Figure 2-2: μ ESI-MS data of 500 nM myoglobin. The myoglobin solution was infused at a rate of 200 nL/min. The peaks are labeled with the number of protons added to the myoglobin macromolecule.

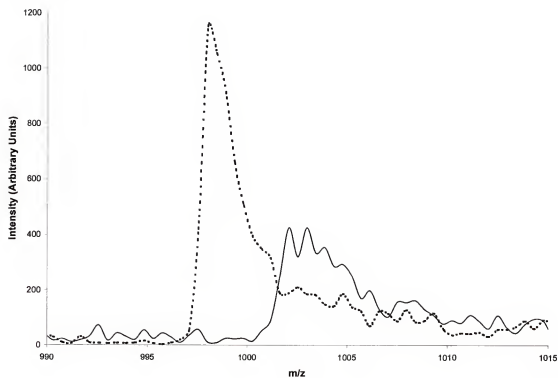


Figure 2-3: μ ESI-MS data of the +17 charge state of myoglobin on the ITMS. The dotted and solid traces are the $t = 0$ and $t = 1$ second reaction times, respectively, of this $(M+17H)^{+17}$ ion with D_2O within the ion trap volume (see text for details).

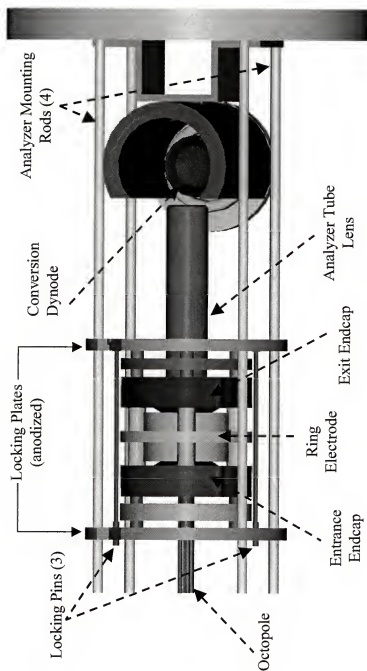


Figure 2-4: 3D AutoCAD drawing of the ESI-ITMS analyzer assembly. Note that the electron multiplier, which is situated orthogonal to the conversion dynode, is omitted from the drawing for clarity.

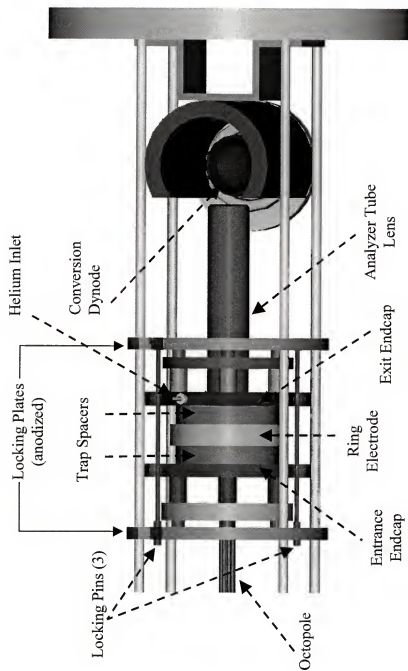


Figure 2-5: 3D AutoCAD drawing of the PHOENIX analyzer assembly. GCQ ion trap electrodes are incorporated into the place where the ITMS electrodes were previously located (Figure 2-4). Note that the GCQ ion trap spacers form an enclosed volume such that the helium buffer gas conductance is much lower than on the open configuration ITMS ion trap.

Exit Endcap, Multiplier View

Material: Stainless Steel

Units: Inches

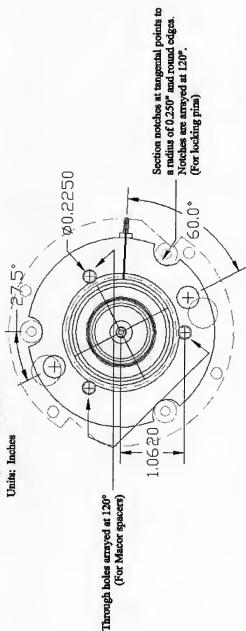


Figure 2-6: 2D AutoCAD drawing of the GCQ endcap electrode on PHOENIX. The dashed component is one of the anodized locking plates that supports the entire analyzer assembly via the locking pins (see Figure 2-5). Hemispherical notches were milled out of the endcap electrodes to prevent obstruction of the locking pins. Also, the endcaps were modified with three arrayed through holes for Macor support spacers. Both exit and entrance endcaps were modified in an analogous manner.

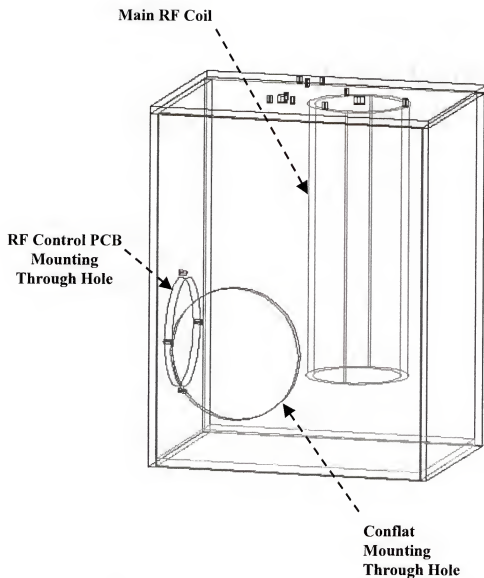


Figure 2-7: 3D wireframe AutoCAD drawing of RF coil box. The viewpoint is from the side that mounts to the vacuum manifold Conflat flange. The dimensions of the aluminum box are 12.235" (h) $\times$ 9.750" (w) $\times$ 7.400" (d).

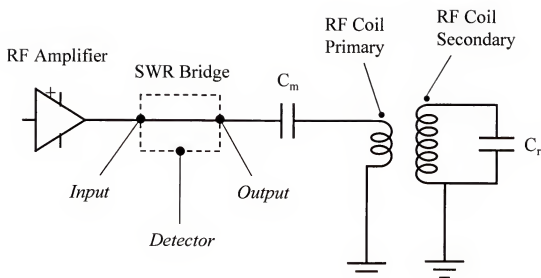


Figure 2-8: Basic schematic of the main RF coil circuit. During RF coil testing, the RF amplifier is disconnected from the circuit and replaced by the SWR bridge. The input, output and detector of the SWR bridge are connected to a waveform generator, the matching capacitor (C_m), and an oscilloscope, respectively. The resonant capacitance (C_r) is due to the ring electrode and the RF coil secondary.

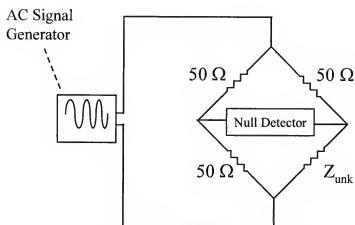


Figure 2-9: The Wheatstone bridge circuit of the SWR measurement device. The input of the SWR bridge is connected to an AC signal generator. The unknown impedance (Z_{unk}) is due to the circuit load (e.g., the RF coil), which is connected to the output of the SWR bridge. An oscilloscope is connected in parallel to the null detector of the SWR bridge to monitor the response from the RF coil as the frequency from the signal generator is swept.

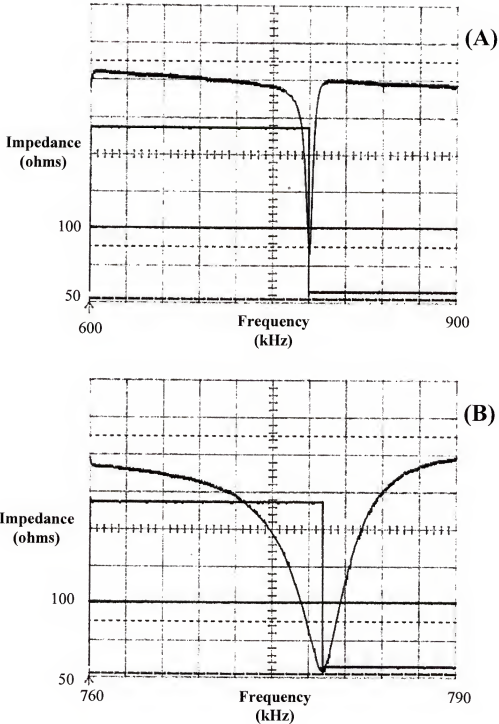


Figure 2-10: SWR signal traces for main RF coil on PHOENIX. Oscilloscope traces display (A) wide frequency range and (B) narrow frequency range swept by the AC signal generator. The marker trace in both displays is set at 779 kHz. As determined from trace (B), the impedance of the load is 55 ohms, resulting in a calculated SWR of 1.1 to 1.0 (see text for details). The discrepancy in the SWR signals between (A) and (B) is due to the large RC time constant of the null detector circuitry of the SWR bridge.

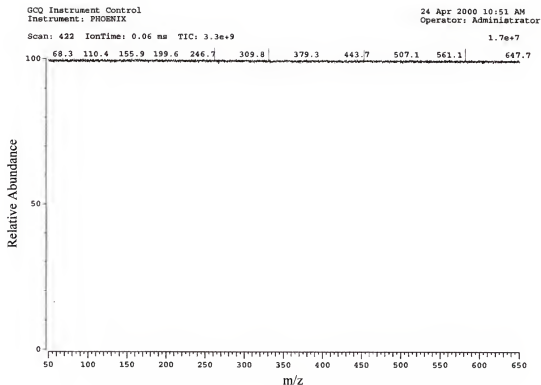


Figure 2-11: Electrometer signal using a coax transmission cable. Even though no ions were being produced, a large electrical signal was observed (Total Ion Current = 3.3×10^9). Disconnecting the coax shield from ground on either end or both ends had no effect on the signal.

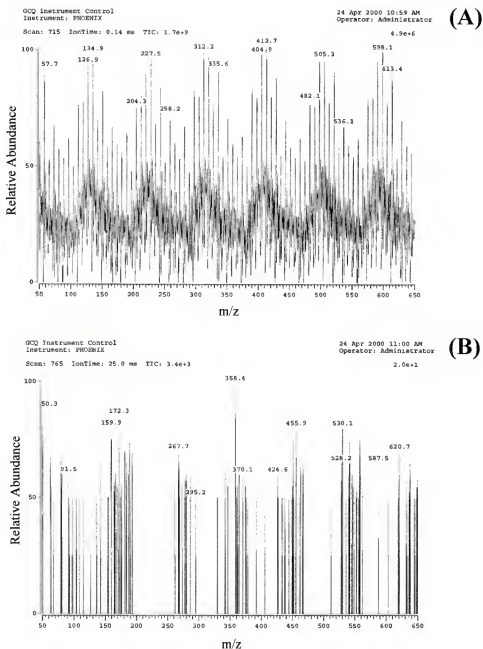


Figure 2-12: Electrometer signal using twisted pair transmission cable. (A) Observed signal when the twisted pair shield is not connected to ground. The AC signal is likely electrical noise from the main RF voltage. (B) Signal observed when the shield is terminated to ground at the electrometer input. Nearly all electronic noise has been eliminated.

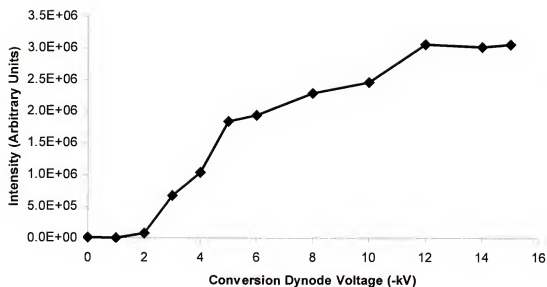


Figure 2-13: The effect of the conversion dynode voltage on ion intensity. Note that the dynode voltages are negative since these data were acquired in the positive ion mode. The intensity of the $(M+H)^+$ ion from Neurotensin (1-6) levels off at dynode settings above -12 kV, permitting the use of the GCQ dynode power supply, which provides a fixed -15 kV (in the positive ion mode), without sacrificing instrument sensitivity.

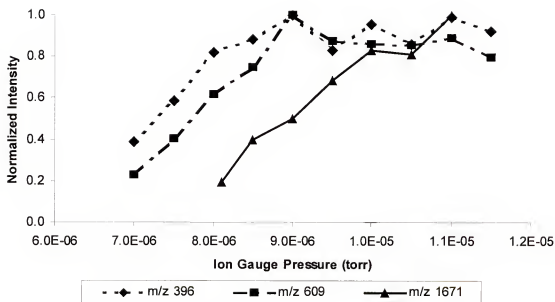


Figure 2-14: Trapping efficiency of externally injected ions as a function of helium pressure. Low m/z ions are effectively trapped at helium bath gas pressures corresponding to 9.0×10^{-6} torr on the analyzer ion gauge. Ions of higher m/z (e.g., $\geq m/z$ 1000) are more effectively trapped at higher helium pressures; however, elevated pressures within the QIT have some deleterious effects (see text for details).

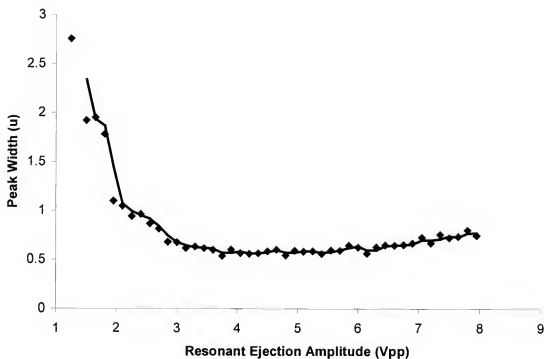


Figure 2-15: Calibration of the resonant ejection amplitude for m/z 396. The m/z -normalized resonant ejection amplitude applied during mass analysis is based on the minimized peak width determined by plots of this type using calibrant ions across a wide m/z range.

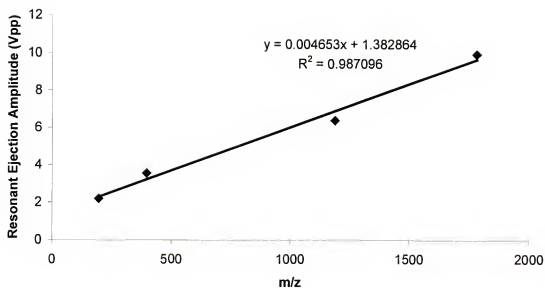


Figure 2-16: Linear regression fit of the m/z -normalized resonant ejection amplitude. The GCQ software applies the linear equation to ramp the resonant ejection amplitude during the ion ejection period of the QITMS scan.

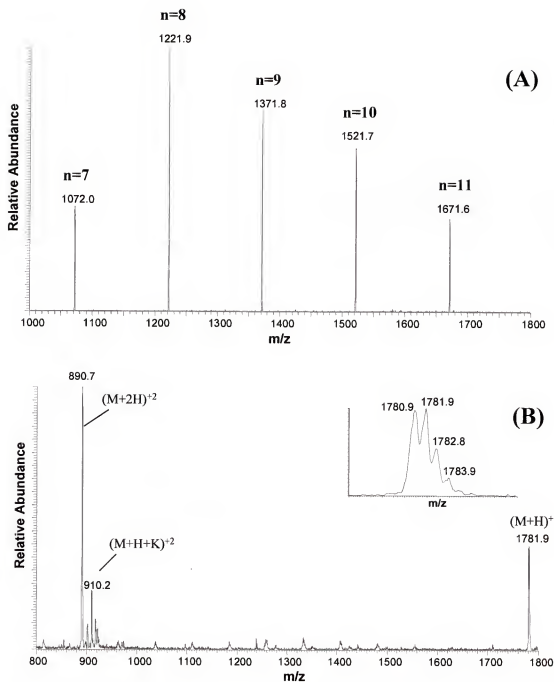


Figure 2-17: μ ESI-MS data demonstrating m/z range on PHOENIX. (A) Mass spectrum of 10 mM sodium iodide in acetonitrile. Ion clusters are of the type $(\text{NaI})_n\text{Na}^+$, where $n = 7-11$ as labeled in (A). (B) Singly and doubly charged ions are observed in the μ ESI mass spectrum of the Sendai virus nucleoprotein fragment (321-336) peptide. The inset displays the isotopes for the $(\text{M}+\text{H})^+$ ion.

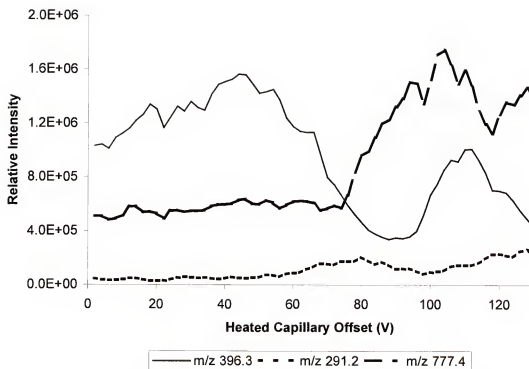


Figure 2-18: Plot of the heated capillary voltage tune data. Ion transmission for low m/z ions is optimized at lower heated capillary potentials, while high m/z ions are transported more efficiently at higher voltages. However, at higher heated capillary offset voltages, ion dissociation of low m/z ions is observed as demonstrated by the increased intensity of the m/z 291 fragment ion produced from the m/z 396 precursor ion ($(M+H)^+$ of spiperone).

Table 2-1: Voltages used for the ion optics on the PHOENIX instrument. The potentials were calibrated and optimized for positive ion μ ESI-MS.

	Functional Voltage Range	Typical Operating Voltage
Heated Capillary	+20 - +85 V	+45 V
ESI Tube Lens (gate open)	+15 - +50 V	+20 V
Skimmer	Ground	Ground
Octopole [DC amplitude]	-2.5 - -3.5 V	-3.2 V
Octopole [AC amplitude]	144 - 241 V _{0-p}	193 V _{0-p}
Ion Trap Offset	Not determined	-7.5 V
Analyzer Tube Lens	0 - +100 V	+50 V

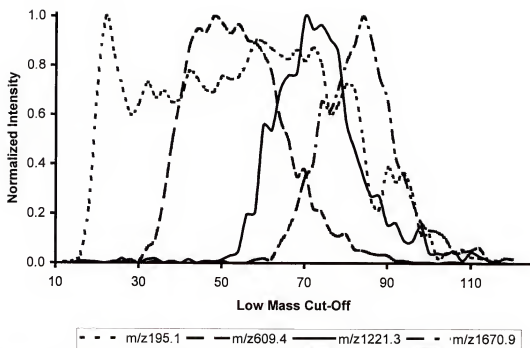


Figure 2-19: Calibration of ion injection as a function of the main RF voltage. The main RF amplitude is plotted in terms of low mass cut-off, as described in the text.

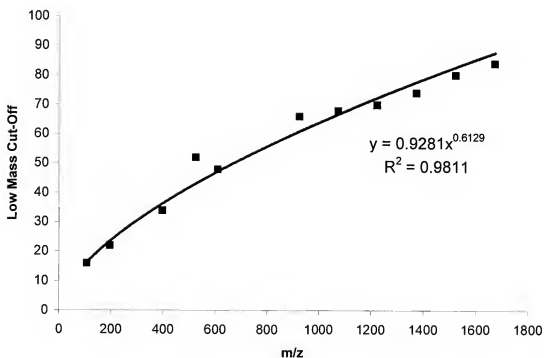


Figure 2-20: Plot of optimal LMCO as a function of ion m/z on PHOENIX. Based on these data, it is necessary to vary the LMCO (i.e., main RF amplitude) during ion injection to prevent m/z bias for μ ESI-MS analyses.

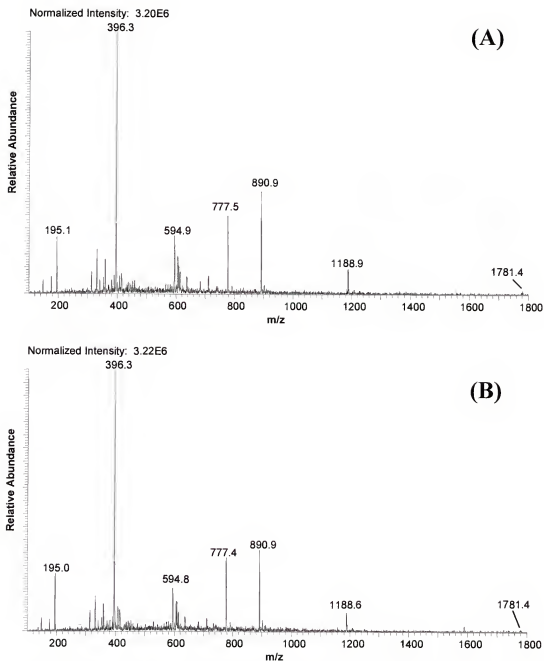


Figure 2-21: Comparison of μ ESI-MS data when varying the main RF amplitude during ion injection. (A) The main RF amplitude is set at two discrete levels corresponding to LMCO values of 36 and 88. (B) The main RF amplitude is linearly ramped with voltages corresponding to LMCO settings from 36 to 88. The observed ions are as follows: caffeine (m/z 195.0); spiperone (m/z 396.3); eleodisin (m/z 594.8 & 1188.6), neurotensin frag. 1-6 (m/z 777.4) and Sendai virus nucleoprotein frag. 321-336 (m/z 890.9 & 1781.4).

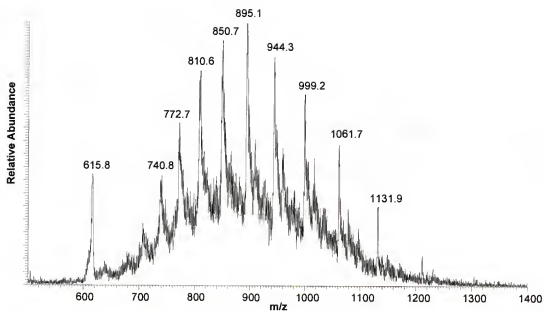


Figure 2-22: μ ESI-MS data of myoglobin acquired with ion gate on PHOENIX closed. The observation of the myoglobin ions (compare to Figure 2-26) when the ESI tube lens (i.e., the gate lens) was at a fixed potential of -130 V indicates that this voltage is not sufficient to prevent transmission of high mass ions to the QITMS.

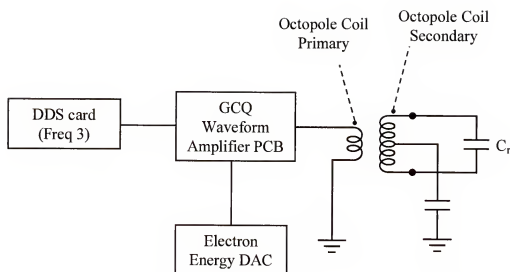


Figure 2-23: Basic schematic of the revised octopole circuit on PHOENIX. The DC offset to the octopole is applied to one side of the octopole coil secondary (omitted from schematic). The resonant capacitance (C_r) is due to the octopole rods and the octopole RF coil. The AC signal to the octopole coil primary is provided by the Dynamic Digital Synthesizer (DDS) card, modulated by the electron energy DAC, and driven by a GCQ Waveform Amplifier PCB. For more information on the operation of the circuit, refer to the text.

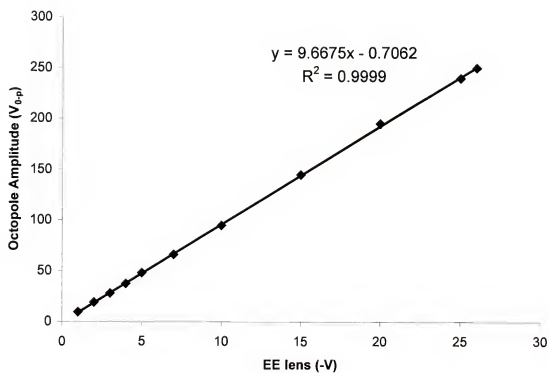


Figure 2-24: Approximate octopole AC amplitude as modulated by the electron energy lens setting. The exact voltages applied to the octopole cannot be determined due to the differences in the capacitive loads between the oscilloscope and the octopole rods.

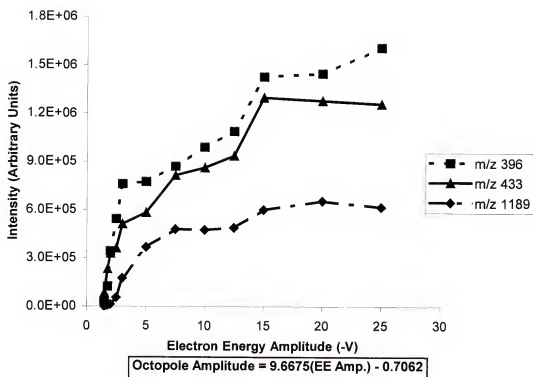


Figure 2-25: Plot of ion intensity as a function of the octopole AC amplitude. The octopole AC amplitude is modulated by the electron energy (EE) voltage setpoint. The optimal ion transmission for a wide range of m/z ions is realized at an EE voltage setting above -15 V (octopole amplitude = $144 V_{0-p}$).

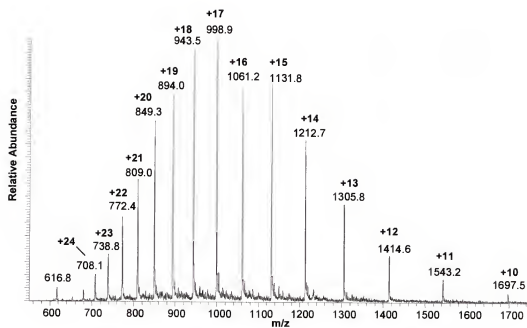


Figure 2-26: μ ESI-MS data of myoglobin with revised octopole circuit. The individual myoglobin ions are labeled with their observed m/z and positive ion charge state (bolded).

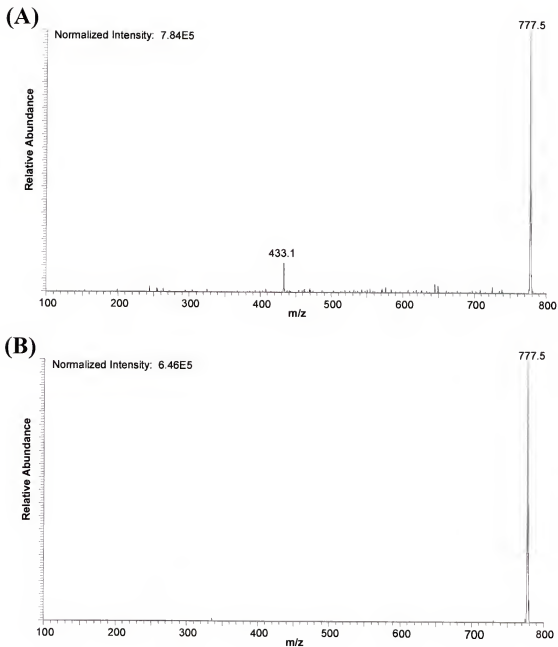


Figure 2-27: Isolation data for m/z 777. (A) Observation of the $(M+3H)^{+3}$ ion of angiotensin I at m/z 433.1 in this isolation mass spectrum indicates that the octopole AC amplitude was not set to zero until after the isolation step. (B) Isolation of the neurotensin 1-6 $(M+H)^{+}$ after changing the GCQ scan matrix to set the octopole AC amplitude to zero after ion injection and prior to ion isolation.

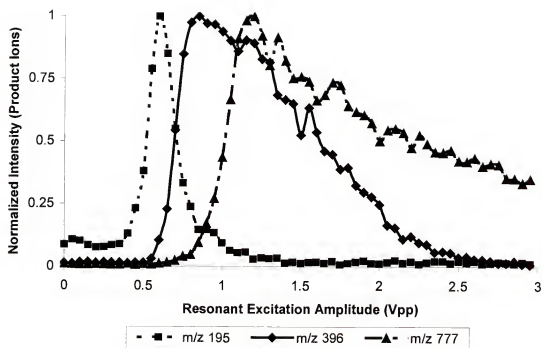


Figure 2-28: Collision-induced dissociation breakdown curves. The normalized intensities of the product ions generated from the listed precursor ions are plotted as a function of the resonant excitation amplitude applied to the endcap electrodes during MS/MS analysis. The precursor ions were resonantly excited at $q_z = 0.225$ for a period of 15 ms.

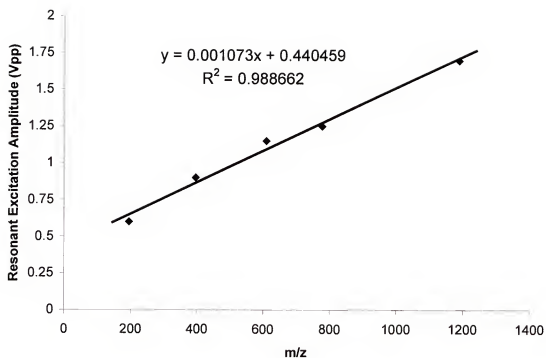


Figure 2-29: Linear calibration of m/z -normalized resonant excitation amplitude. The optimal resonant excitation amplitude generated from CID breakdown curves (see Figure 2-28) are used to generate this plot.

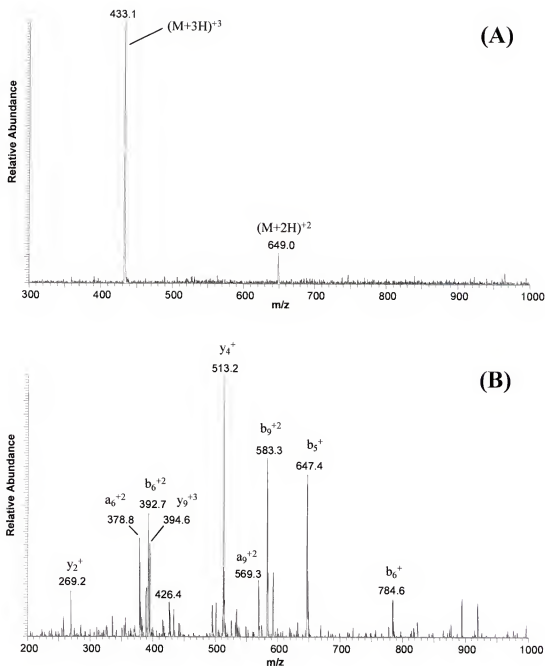


Figure 2-30: Representative mass spectra for 1 μ M angiotensin I. (A) μ ESI-MS data at 200 nL/min. displaying the doubly and triply protonated angiotensin I ions. (B) μ ESI-MS/MS data from CID of m/z 433 yielding product ions consistent with the sequence of angiotensin I (DRVYIHPFHL). The angiotensin I product ions are labeled using the Biemann nomenclature for peptide ions.¹⁴⁸

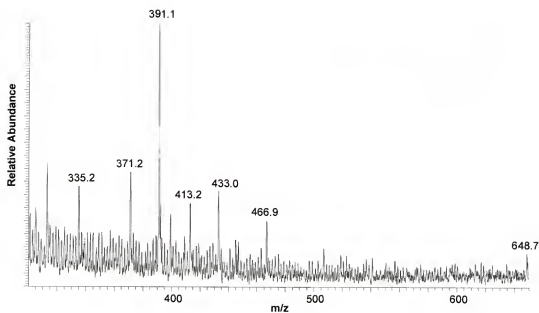


Figure 2-31: μ ESI-MS data of 10 nM angiotensin I. The $(M+3H)^{+3}$ can be observed at m/z 433 at a signal-to-noise of 2:1. These data represent a consumption of 12 attomoles of angiotensin I.

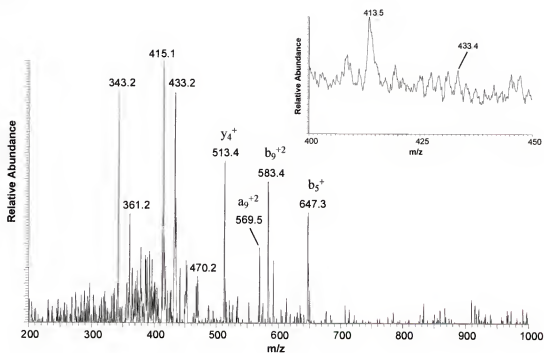


Figure 2-32: μ ESI-MS/MS data for the m/z 433 from 1 nM angiotensin I. The product ions from the $(M+3H)^{+3}$ of angiotensin I are labeled with the Biemann nomenclature. These data represent the consumption of 30 attomoles of angiotensin I. The inset is the μ ESI-MS data produced from this sample. Notice that the m/z 433 ion is obscured by the chemical background.

CHAPTER 3 INCORPORATION OF CAPILLARY LIQUID CHROMATOGRAPHY

Introduction to cLC/MS

Chromatographic separation of mixtures has been an integral part of the chemical sciences since its discovery by Russian botanist Mikhail Tswett in 1906.¹⁴⁹ Over the past 25 years, efforts in developing microscale liquid chromatography¹⁵⁰⁻¹⁵⁵ have led to significant advances in lowering the limits of detection for analytical instrumentation. The primary advantage of liquid chromatography is that the concentration factor of separated components is inversely proportional to the square of the internal diameter (I.D.) of the column.¹⁵⁶ In other words, small decreases in the internal diameters of LC columns lead to larger increases in the concentration of analytes eluting from the column. Therefore, capillary LC columns (i.e., columns with internal diameters less than 500 μm) yield the best sensitivity for compound detection. However, a drawback of reduced I.D. columns is that correspondingly less sample can be applied to the LC column before overloading effects are observed and the separation ability becomes compromised.

The on-line coupling of liquid chromatography to mass spectrometry has undergone significant development since the initial reports in the early 1970s.¹⁵⁷⁻¹⁶⁰ The major difficulty of interfacing LC with MS is the strain placed on the vacuum system by the mass load of the liquid as it is introduced into the mass spectrometer and vaporized. Therefore, the solvent must be eliminated prior to the high vacuum region of the mass spectrometer. Also, many of the analytes typically separated by LC are nonvolatile

and/or thermally labile, and the ionization methods used with mass spectrometry prior to circa 1980 were not compatible with these compounds. The advent of new ionization methods, such as thermospray¹⁶¹ and fast atom bombardment (FAB),¹⁶² that produce gas-phase ions from thermally labile compounds provided suitable means of coupling LC with MS. While LC/MS using thermospray ionization was widely used in the 1980s,¹⁶³ the FAB interfaces for LC applications achieved limited acceptance; however, cLC/MS was accomplished using a continuous-flow FAB source.¹⁶⁴ Some other LC/MS interfaces that were devised in the 1970s and early 1980s included particle beam,¹⁶⁵ moving belt,^{160,166} and direct liquid introduction,^{157,158,167} each with limited success. The advent of electrospray ionization^{65,66} truly led to the LC/MS revolution.

During the early years of ESI development, the ESI sources were limited to flow rates between 1 and 20 $\mu\text{L}/\text{min}$. and therefore could be directly coupled to "larger-bore" capillary LC columns (e.g., 250-500 μm I.D.) as demonstrated by Huang and Henion in 1991.¹⁶⁸ When it was established that ESI-MS acts as a concentration sensitive device,¹⁰⁰ more investigators began to apply cLC to ESI-MS to maximize the mass and concentration sensitivity of analyses. Once nanobore capillary columns (I.D.s ≤ 150 μm)¹⁶⁹ were employed for cLC/ESI-MS, researchers required the use of a sheath liquid as a "make-up" flow for the ESI source^{106,107,170,171} since the flow rates compatible with these cLC columns were too low to generate a stable electrospray. The use of a sheath liquid causes dilution of the eluting components from the capillary column and decreases the sensitivity of cLC-ESI/MS analyses. The development of microelectrospray ionization (μESI) interfaces⁷⁶⁻⁷⁹ overcame this obstacle and allowed true coupling of nanobore cLC/MS^{80,103,172-176} using capillary columns as small as 25 μm I.D.¹⁴²

Currently, cLC/ μ ESI-MS methods are widely used for peptide mapping and sequencing as part of the growing field of proteomics.¹⁷⁷ The high selectivity and sensitivity of cLC/MS(MS) can provide unprecedented qualitative and quantitative information on complex mixtures. Thus, as improvements are continually made in both the mass spectrometric and chromatographic technologies, cLC/MS will continue to push the mass limits of detection beyond the currently reported low attomole^{141,142,176} and high zeptomole levels¹⁴³ and make an even greater impact in the biological sciences.

Construction of Capillary Columns

High-Pressure Packing Chamber

As is the common practice, the capillary LC columns were created by packing the stationary phase into fused silica (f.s.) capillary tubing. To accomplish this task, a device that provides pressures on the order of 1000-3000 pounds per square inch (psi) is required to physically force the fine stationary phase particles into the f.s. capillaries. The two devices most utilized are an HPLC pump or an inert gas pressurized chamber. For the experiments described here, the latter apparatus was chosen to fabricate the cLC columns.

Construction of the high-pressure chamber (i.e., bomb) required careful consideration of the safety factors involved with using a device designed to provide extraordinary high forces (e.g., 1000 psi acting on an area of a 365 μ m O.D. capillary). Accordingly, the high-pressure bomb was created from stainless steel (SS) employing an o-ring compression seal with six 1/4"-28 bolts through the lid to the body to ensure a tight, secure seal. The three-dimensional wireframe drawing for this apparatus is given in Figure 3-1. The body of the SS bomb was hollowed out to a diameter slightly larger than the outer diameter of a 1/2-dram vial, to which the stationary phase slurry was added

during the capillary packing process. The through hole near the bottom of the base was tapped for a 1/16" NPT to 1/8" Swagelok fitting through which the SS chamber could be pressurized. To feed the f.s. tubing into the bomb, a Valco SS union with a 0.5 mm bore was welded into the center of the lid.

To pressurize the SS bomb, a regulator rated to a maximum pressure of 4000 psi and a three-way SS valve were acquired. The regulator was connected to a tank of high purity (99.8%) helium with the three-way SS ball valve attached to the output of the regulator. The other two channels of the valve were connected to the SS bomb via 1/8" SS tubing and to a PEEK tubing restrictor (Figure 3-2). The three-way valve was employed to permit the three modes of operation: helium flow termination, pressurization of the SS bomb, and venting the bomb to atmosphere. To ensure the integrity of the SS chamber and various connections, the high-pressure apparatus was tested at 2000 psi for a period of one hour. The pressure gauge was monitored with the helium regulator closed for signs of pressure loss and the connections were checked for leaks, of which neither was found.

Initial Capillary LC Columns

When chromatographic experiments are conducted on reduced diameter columns, additional care must be given to eliminating extracolumn volume, particularly postcolumn volume. As the column diameter is reduced, the elution volume containing the analyte decreases rapidly (i.e., as the square of the internal diameter of the column). Therefore, when nanobore capillary columns are employed, where elution volumes are on the order of tens of nanoliters, it is imperative to have the detector as close to the capillary column output as possible to prevent significant postcolumn band broadening.

Since even “zero dead volume” unions have dead volumes comparable to the elution volumes on nanobore cLC columns, it was decided that the stationary phase should be packed into the same f.s. capillary in which the μ ESI emitter was fashioned.

The first method explored to create a unified cLC column/ μ ESI emitter involved packing a short region of the tapered end of the f.s. capillary with large porous particles followed by a common silica-based stationary phase. The hypothesis is that the porous particles, having diameters larger than the I.D. of the tapered μ ESI emitter, would create a suitable frit whereby the stationary phase could be retained and the mobile phase could easily pass through to form the electrospray.¹⁷⁵ Fused silica capillary tubing (365 μ m O.D., 50 μ m I.D.) cut into lengths of 30-36 inches was used to produce two μ ESI emitters of approximately equal length tapered to about 5 μ m I.D. with a Sutter P-2000 laser micropipette puller. The distal end of the tapered f.s. capillary was inserted into the SS bomb containing a 4-6 mg/mL slurry of stationary phase particles, which was agitated with magnetic stirring (see Figure 3-2). The “fritting” slurry was a solution of 10 μ m POROS particles, a polymeric particle with high porosity. This slurry of POROS particles was briefly pressurized to 250-400 psi until approximately 1-2 cm of the tapered end of the f.s. capillary was packed. The SS bomb was then slowly depressurized and a vial containing a slurry of 5 μ m particles modified with C_{18} stationary phase at 4-6 mg/mL in 60/40 (% v/v) 2-propanol/acetonitrile was inserted into the high-pressure device. The fritted capillary was secured and the bomb was repressurized to 1000 psi. Visual inspection confirmed that the C_{18} particles were filling the capillary; however, after reaching a packing length of 3-5 cm the packing process ceased. Increasing the

bomb pressure up to 1500 psi had no effect on reinitializing the capillary packing. This method of creating cLC columns was attempted several times, each ending with the same results: the C_{18} particles stopped filling the capillary after reaching a length of 3-6 cm with no solvent flowing from the tapered output.

Since the process of forming the combined porous particle/silica particle columns failed, attempts were made where only POROS particles were packed into the tapered fused silica capillaries. Several trials did produce a couple of POROS packed columns with bed lengths between 10 and 15 cm. Nevertheless, during flushing of the columns with 80% methanol, the solvent flow ceased after 30-90 minutes, rendering these columns useless.

With the assistance of an optical microscope, the columns created using these two methods were visually inspected, which revealed the problem. A single POROS particle was being lodged into the mouth of the approximately 5 μm I.D. capillary taper. This apparently caused liquid flow about the POROS particle impossible and flow through the particle was too restrictive to attain sufficient flow rates to observe solvent droplets from the outlet end of the capillary. Hence, a new method of preparing a unified capillary column/ μESI emitter was required.

Preparation of Porous Frits

With the lack of success in producing usable capillary LC columns within the μESI emitter capillary, alternatives for creating frits within the f.s. capillary were investigated. A common means of forming a porous glass frit for packing cLC columns is to sinter into the outlet end of the capillary a small length of silica beads.¹⁵⁵ However, this requires tapping the f.s. capillary into a pile of silica particles, which would likely

damage the tapered μ ESI emitter tip. Another possibility was to insert a commercially available hydrophilic PVDF membrane frit into the μ ESI emitter from the distal end, as described by Davis et al.⁸⁰ The problem with this technique was that the I.D. of the capillaries used in this research project (50 μ m) was significantly smaller than that used by Davis and co-workers (150 μ m), making the introduction of such a frit into these f.s. capillaries highly problematic.

A promising method of fashioning semi-permeable frit into the μ ESI emitters was one presented by the Hunt group at the ASMS conference in 1998.¹⁷⁸ In this method, an aqueous solution of potassium silicate, commercially called Kasil, was drawn by capillary action into the end of a f.s. capillary and allowed to form a porous crystalline glass frit. A 30% (w/v) Kasil solution was obtained for trial experiments to develop the methodology for producing viable frits for packing capillary columns. This highly viscous solution did not produce usable frits when allowed to crystallize at room temperature, even when the solution was first diluted with water. Subsequently, attempts were made to "cure" the glass frits at elevated temperatures within a GC oven. Using temperatures below 100° C still did not crystallize the Kasil plug in the capillaries, even after several days of treatment. At temperatures above 100° C the solvent was driven away; however, what remained in the f.s. capillary was a solid glass plug that would not permit solvent to pass through. It seemed that an exacting set of experimental parameters (e.g., potassium silicate concentration, oven temperature, crystallization time) would be necessary to form porous glass frits from the Kasil solution; perfecting this methodology

could have taken weeks and wasted a great deal of fused silica tubing. Therefore, this process was also abandoned.

The recent development of monolithic columns,¹⁷⁹ where the stationary phase is a composed of a homogeneous material synthesized within the column, offers another possible approach. These polymer-based or silica-based monoliths have the advantage that the chemical nature and porosity of these columns can be altered to fit the needed application. Since these monolithic columns are created from either a monomeric solution for polymerization or from alkoxide-based silica gels, the creation of a frit using this type of process would be advantageous. Indeed, the procedure described above using the Kasil solution was an attempt at synthesizing a silica-based monolithic frit. Fortunately, the Kennedy group with their recent cLC/MS work had already developed a method for producing a polymeric-based monolith for the purpose of creating capillary column frits,¹⁴² which they graciously shared with the author.

The polymeric frit is synthesized via photopolymerization with ultraviolet (UV) radiation. In the interest of confidentiality, the specific compounds are not given here. The basics of the process are to prepare a co-monomer solution containing an initiator, fill the capillaries with the solution, irradiate the capillaries with a UV lamp for 30 minutes, and then flush the unused solution from the f.s. capillaries. The advantage of this method is that the frit is UV photopolymerized, which permits selective positioning of the frit in the f.s. capillaries. The polyimide cladding must be removed to permit transmission of the UV light to the co-monomer solution for polymerization. Hence, a small window, 2-4 mm in length, is created by a miniature electrical arcing apparatus approximately 3 inches upstream to where the μ ESI emitter is later processed via the

laser puller. These photopolymer frits are highly porous yet withstand pressures up to at least 3000 psi without any detrimental effects.

After the capillaries have been flushed of the unused co-monomer solution with acetone and subsequently purged with helium, the μ ESI emitter tips are produced. This is accomplished by inserting the f.s. capillary into the Sutter P-2000 micropipette puller until the frit window resides just outside the laser encasement piece. The optimal parameters for fashioning μ ESI emitters with I.D.s approximately 4-6 μ m from 365 μ m O.D., 50 μ m I.D. f.s. capillaries are as follows: heat 350, filament 0, velocity 9, delay 160, pull 10. The cycle may need to be repeated 2-3 times to finish the pulling process. No further treatment (e.g., etching with hydrofluoric acid) of the emitters was required prior to use when these programmed parameters were utilized.

Once the f.s. capillaries were fritted and tapered, the stationary phase was packed into the capillary tubing. As before, a 4-6 mg/mL slurry of 5 μ m C_{18} particles in 60/40 2-propanol/acetonitrile was prepared in a 1/2-dram vial and added to the SS bomb with magnetic stirring. The distal end of the fritted μ ESI emitter was inserted into the slurry solution through the lid of the SS bomb and secured by the Valco union. After bolting the lid to the body of the SS bomb, packing was initiated by opening the three-way valve to the helium tank. Once it was confirmed by visual inspection that the silica particles were filling the capillary, the pressure was slowly increased to 1500 psi. All columns prepared by this method were packed to a length of about 10 cm, which required between 20 and 30 minutes. To prevent regression of the stationary phase toward the inlet end of the capillary, the SS bomb was allowed to depressurize slowly over a period of 30

minutes. For columns that were to be immediately used, the capillary was connected to an LC pump and flushed with water/methanol for a minimum of 1 hour. If the cLC columns were to be stored for later use, the capillaries were purged of any remaining solvent with helium using the SS bomb.

cLC/MS Apparatus

Using capillary columns with I.D.s of 50 μm requires flow rates on the order of 100-200 nL/min. to produce optimal chromatographic results. Since current LC pumps cannot adequately operate at such low flow rates, a means of splitting a majority of the LC effluent from the LC pump was required. If this was a matter of dividing liquid flow between two open-tubular channels, a simple equation could be employed to calculate the necessary dimensions for a diverting tube to provide the proper fractional flow to the LC column. However, the cLC column is filled with silica particles creating an additional resistance to flow that cannot be easily determined with mathematical equations. Therefore, experimental trial and error was used to reduce the capillary column flow rate into the targeted range. Determination of the flow rate through the cLC column by temporal measurement of a fixed volumetric flow through the waste line was helpful but imprecise due to the large split ratios and to the loss of solvent during the measurement. Ultimately, another method of calculating the capillary column flow rate would be accomplished via mass spectrometric detection of an unretained compound injected into the LC flow stream (see below).

The desired flow split was realized with a 0.75 mm bore Valco tee to which the LC effluent, provided by a Waters 600E dual reciprocating pump, was partitioned by a 100 μm I.D. f.s. capillary with lengths between 35 and 100 cm. The cLC column/ μESI

emitter was connected to the third port of the tee and secured to the μ ESI interface via the Delrin probe as described in Chapter 2. A block diagram illustrating the cLC/ μ ESI-MS setup is given in Figure 3-3. Since the μ ESI emitter is composed of fused silica, which is an electrical insulator, the electrospray high voltage was applied at the splitting tee. To prevent possible current migration away from the μ ESI source, the LC pump was grounded at the SS fitting on the PEEK delivery tubing.

The first objective with a new capillary column/ μ ESI emitter was to establish a stable ESI-MS signal. Initially, this task was accomplished by adding the well-characterized organic compound spiperone at 0.5 ng/ μ L to the “strong” mobile phase reservoir, namely 80% methanol with 0.15% formic acid (FA), which was continuously pumped through the cLC column. At the high organic content of the mobile phase, the spiperone would not be sufficiently retained on the capillary column and therefore a constant concentration of the analyte would be provided to the μ ESI source for the purpose of optimizing the $(M+H)^+$ signal via adjustment of the μ ESI emitter position and ESI high voltage. This method, while effective for tuning, was quickly discarded due to the fact that the spiperone required flushing from not only the capillary column but also from the LC pump transfer lines and seals prior to sample analyses.

The revised method of tuning the μ ESI-MS signal was to incorporate a large volume sample loop in series with the LC pump effluent. A two-way Rheodyne 6-port injection valve with a 2.3 mL loop was added to the LC flow stream prior to the splitting tee. Advantages of tuning the μ ESI source in this manner are as follows: the injected sample does not contaminate LC pump as before; the known swept volume between the

loop and the μ ESI emitter and the known LC pump flow rate allow the facile calculation of the flow rate through the cLC column (see below); the large volume provides a sample “plug” lasting several minutes; and the large split ratio diverts most of the sample away from the capillary column while not affecting the ion signal due to the concentration-sensitive property of ESI-MS. Injection of 100 parts per billion (ppb) spiperone into the flow stream of 100% mobile phase B, typically 90% methanol plus 0.15% FA, was the standard means by which the instrument was tuned on a day-to-day basis.

The volumetric flow rate through the capillary column was determined during the tuning process by measuring the time between the sample injection and the first appearance of an ion signal from the unretained spiperone sample. Since the time delay between the sample loop and the μ ESI emitter is mainly attributed to the f.s. capillary after the splitting tee (due to the high LC pump flow rates and low swept volume of the PEEK tubing between the loop and the tee), the cLC column flow rate is simply the volume of the column f.s. tubing divided by the time for the unretained analyte to pass through the capillary. Such a calculation can be accomplished with the assistance of Figure 3-4, which displays the ion chromatograms for m/z 396 and m/z 185 for a loop injection of spiperone. During this acquisition the spiperone sample was injected at 0.28 minutes and the $(M+H)^+$ ion at m/z 396 first appears at approximately 4.53 minutes. However, the indeterminate background ion at m/z 185 is observed at 4.28 minutes, suggesting that spiperone is retained somewhat as it passes through the capillary column. Hence, the migration time of an unretained component, for example the compound producing the m/z 185 ion, is 4.00 minutes. In this case the length of the 50 μ m I.D. f.s. capillary containing the stationary phase is 14.0 inches. With a volume of 50 nL/inch for

the unpacked 50 μm I.D. capillary, the total volume of the cLC column/ μESI emitter tubing is 700 nL. Therefore, the calculated flow rate through the capillary column is $700 \text{ nL}/4.00 \text{ min.} = 175 \text{ nL/min.}$ It should be noted that the volume due to the stationary phase was not accounted for in this calculation; therefore, the determined cLC column flow rate is an estimate and the true flow rate is somewhat lower than the calculated flow rate. For all cLC/MS(/MS) data presented herein, the LC pump flow rate was adjusted to provide a calculated capillary column flow rate between 100 and 200 nL/min. as determined above.

During the process of tuning the μESI -MS signal, the two primary factors adjusted, the ESI high voltage and the μESI emitter/heated capillary distance, had very important consequences on the signal stability and quality. The distance between the μESI emitter tip and the counter electrode (the heated capillary) played an important role in the ESI high voltage required to maintain an electrospray as well as the appearance of the ESI-MS signal, particularly when the mobile phase had a high aqueous content. Visualization of the emitter/capillary distance was not available with the μESI interface used in this work; therefore, the distance between the emitter tip and the heated capillary was estimated by measuring the lateral translation per turn on the XYZ stage. When the μESI emitter/heated capillary distance was less than approximately 1 mm, the background ESI mass spectrum of a high aqueous content mobile phase displayed cluster ions with repeating units of 74 u (e.g., m/z 355, 429, 503) and their analogous oxidation products at 16 u higher (e.g., m/z 371, 445, 519) with essentially no other background ions (Figure 3-5A). However, if the Delrin probe holding the μESI emitter was moved

back such that the emitter/capillary distance was greater than 1 mm, the ESI-MS data in Figure 3-5B are observed, where the cluster ions are less pronounced and chemical noise ions are seen at nearly every m/z . The hypothesis for this phenomenon is that at small (<1 mm) distances between the emitter tip and heated capillary, corona discharge is the dominant process creating the spectrum in Figure 3-5A, which is more facile with solutions at a high aqueous content due to the high dielectric constant and surface tension of water. If larger emitter/capillary distances are employed (Figure 3-5B), formation of an electrospray is evident due to the reduction in the intensity of those cluster ions produced by corona discharge and a concomitant observation of ubiquitous chemical noise in the mass spectrum. This premise was substantiated by the fact that the $(M+H)^+$ from neurotensin fragment 1-6 was not observed during a loop injection using the conditions in Figure 3-5A, but it was readily seen using the parameters in Figure 3-5B (data not shown).

With the greater distances required between the μ ESI emitter and heated capillary, a higher voltage needed to be applied to the SS tee to establish an electrospray. Unfortunately, the higher ESI voltages provided the basis for generating more electrochemical byproducts and hence a higher chemical background. This effect is demonstrated in Figure 3-6, where the ESI voltage is varied from +4.3 kV to +1.6 kV. The signal from the $(M+H)^+$ ion of neurotensin 1-6 at m/z 777 remains relatively constant until approaching the electrospray onset voltage of +1.6 kV; however, the signal from the background ion at m/z 371 steadily decreases as the ESI voltage is lowered (Figure 3-6). From these data, the lowest possible ESI voltage that generates a constant signal for the analyte should be used to maximize the S/N for ESI-MS analyses. The difficulty is

choosing the proper ESI voltage to use during cLC/MS acquisitions as the onset ESI voltage changes with the mobile phase composition. Since the onset voltage for ESI is greater for water than other organic solvents due to its higher surface tension, the ESI high voltage was tuned via injection of neurotensin 1-6 into a stream of 25% water, 75% methanol. The ESI voltage applied for cLC/MS experiments was 200-500 V higher than the electrospray onset potential.

cLC/MS of Tryptic Digest and Other Peptide Standards

Initial cLC/MS Experiments

Preliminary cLC/MS experiments were conducted using a simple peptide mixture. The purpose of these experiments was to test the separation capability of the capillary column based on the different chromatographic conditions (e.g., gradient ramp rate). The list of peptides in the standard test mixture, which includes a number of FMRFamide-like peptides (FLPs), and their observed ions in the subsequent mass spectra, is given in Table 3-1.

An example of the early cLC/ μ ESI-MS results is given in Figure 3-7. Some of the experimental parameters are as follows: peptide concentration = 5 nM; estimated volume injected = 3-5 μ L; mobile phase A = 5% methanol + 0.15% FA; mobile phase B = 90% methanol + 0.15% FA; LC gradient—linearly ramp from 1% B to 21% B in 4 minutes, hold at 21% B for 5 minutes, ramp linearly from 21% B to 99% B over 39 minutes (i.e., 2% per min.). Under these chromatographic conditions, five of the seven peptides were observed, all of which were well separated (Figure 3-7). Note that the component observed in the m/z 796.5 ion chromatogram eluting at 49.97 minutes is not

consistent with [Lys³]-bombesin as the mass spectrum indicates that this peak is due to an isotope of a sodium-bound dimer ion (data not shown).

In an effort to detect the two missing peptides from the cLC/MS analysis displayed above, it was anticipated that a shallower gradient would successfully chromatograph [Lys³]-bombesin and eleodoisin for positive identification. However, upon inspection of the data in Figure 3-8, in which a linear ramp of 1% per min. was employed after reaching 21% B, only four of the peptides in the standard mixture (Table 3-1) were identified. The two early eluting peptides, neurotensin 1-6 and GFGDEMamide, yield a strong response by cLC/ μ ESI-MS using both the 1% per min. (Figure 3-8) and 2% per min. (Figure 3-7) gradients. In the case of the 1% per min. gradient of Figure 3-8, the two observed later eluting peptides ($\approx$ 43.4 min.) show severe band broadening. At the time it was concluded that this was due to the inadequate ability of the modifier formic acid to sufficiently retain and properly chromatograph these peptides.

Based on these preliminary cLC/MS results, it was decided to attempt chromatographic separation of this standard peptide mixture using trifluoroacetic acid (TFA) rather than formic acid (FA) as the mobile phase modifier. Separation of peptides by reverse-phase liquid chromatography is commonly performed with the use of TFA as the modifier in the mobile phase due to the ion pairing characteristics of TFA. It was believed that the use of TFA would improve the retention of the larger positively charged peptides (since the pH is less than or equal to the pK_a of the peptides) that had not yet been observed by cLC/MS and would increase the resolution of the later eluting compounds by changing the chromatographic mechanism from strictly hydrophobic

interactions of the peptide side chains with the C₁₈ stationary phase to a combined hydrophobic interaction/ion pairing separation.

The cLC/MS results when 5 mM TFA was employed as the mobile phase modifier and using the same gradient as in Figure 3-7 (i.e., 2% per min.) are given in Figure 3-9. There are several notable differences between the cLC/MS analyses using FA (Figure 3-7) and TFA (Figure 3-9). First, the chromatography has been altered as the angiotensin I peak (33.79 min.) elutes after SDPFLRFamide and ASGDPNFLRFamide, which coelute at 32.37 min. in the TFA results (Figure 3-9). Second, the two early eluting peptides (≤ 20 min.) in Figure 3-9 display significant band broadening as compared to the results using a FA modifier (Figures 3-7 and 3-8). Initially it was thought that this consequence was due to the addition of TFA to the mobile phase; however, later results using FA also showed broadened peak shapes (see discussion below). Third, the concentration of the standard peptide mixture (Table 3-1) was increased from 5 nM to 25 nM when using the TFA modifier. This is due to the fact that cLC/MS data with adequate S/N could not be obtained when the lower concentration peptide sample was injected (data not shown). This loss in sensitivity may also address the differences observed in the resultant μ ESI-MS in the TFA-based separations.

Comparison of the angiotensin I mass spectrum from the cLC acquisition using TFA (Figure 3-10A) to that using FA as the modifier (Figure 3-10B) shows apparent differences upon visual inspection. The spectrum in Figure 3-10A presents a prominent ion at m/z 649.1, corresponding to the $(M+2H)^{+2}$ of angiotensin I, while Figure 3-10B displays a base peak at m/z 433.3, which is the $(M+3H)^{+3}$ of angiotensin I. These differences can be attributed to the strong ion-pairing property of TFA, which impedes

the peptides from migrating to the surface of the charged electrosprayed droplets. Since the analyte ions cannot reach the droplet surface until later-generation progeny droplets fissure from the main electrosprayed droplet, the charge neutralization effect of TFA causes a delay in the liberation of gas-phase ions resulting in a decrease in ESI-MS sensitivity. TFA is a stronger acid than FA ($pK_a = 0.3$ and 3.75 , respectively) and therefore was anticipated to have produced a prevalent $(M+3H)^{+3}$ ion for angiotensin I in the ESI mass spectrum at the TFA concentration used. However, the effect of the delay in analyte ion migration by TFA to a droplet's surface is that a lesser amount of charge remains on these electrosprayed droplets as the early progeny droplets contain most of the excess charge generated during ESI (see Chapter 1).⁹² This ion-suppression characteristic of TFA on ESI-MS has been reported previously in the literature¹⁸⁰⁻¹⁸² and is the reason that TFA is rarely used with LC/MS. Finally, and perhaps most notably, [Lys³]-bombesin and eledoisin in the standard peptide mixture (Table 3-1) were still not observed when the TFA modifier was used. The lack of significant improvement in chromatographic performance coupled with the detrimental effects observed with the TFA modifier resulted in the return to using FA as the mobile phase modifier in subsequent cLC/MS experiments.

The inability to positively identify all the components of the standard peptide mixture does not appear, based on these data, to be a function of the mobile phase modifier. In retrospect, the lack of success appears to have been due to the use of gradients that ramped too slowly, leading to the loss of chromatographic concentration as the band moved down the capillary column. Discussions with members of the Kennedy group were constructive in suggesting the use of a steeper gradient to optimize the

chromatographic performance. Modification of the cLC/MS methodology to adopt a more rapid gradient is presented in the next section.

Revised Injection Apparatus

Despite the difficulty in the preliminary cLC/MS experiments to observe all the peptides in the standard mixture, another matter that needed to be addressed was the method of sample injection. By employing the SS bomb to load samples onto column, two significant problems were encountered. One, the f.s. capillary was fractured on a couple of occasions when the PEEK sleeve was retracted prior to the capillary's insertion into the bomb's lid. Two, there was no accurate way of determining the amount of sample injected on the capillary column. Therefore, an alternate injection scheme was devised for delivering a known volume of sample to the column for cLC/MS analyses.

The new method for sample injection uses a two-way, six-port Rheodyne injector that is connected to an isocratic LC pump via a Valco spitting tee, as illustrated in Figure 3-11. This injection apparatus is off-line from the cLC/MS setup (Figure 3-3) for two reasons. First, an on-line injection system would require as little extracolumn volume as possible to prohibit extremely long lag times between the gradient's initial formation point and its arrival at the capillary column. As such, a special low-volume injector would have had to been purchased at a significant cost. Second, the off-line setup provides flexibility not easily attained with an on-line injection scheme. For instance, samples can be loaded onto the capillary column/ μ ESI emitter using significantly larger injection volumes at elevated flow rates. For the apparatus used in this research, an injection loop of 5 μ L was utilized. Samples were loaded using a mobile phase of 5%

methanol plus 0.15% FA (i.e., eluent A) at a pressure of 3000-3500 psi, which equated to an approximate volumetric flow rate of 1 $\mu\text{L}/\text{min}$.

To test the feasibility of the new injection setup, the standard peptide mixture (Table 3-1) at a concentration of 25 nM was loaded onto the capillary column. After a period of desalting and equilibration on the cLC/MS system at 99% eluent A (5% methanol + 0.15% FA), the analysis was initiated. As mentioned above, a more rapid gradient of 4% per minute was employed to improve the chromatographic performance. The gradient was programmed to linearly ramp from 99% A to 99% B (90% methanol + 0.15% FA) in 24.5 minutes. Figure 3-12 demonstrates the performance using the steeper gradient. The most notable observation is that all seven peptides in the standard mixture are detected with this method. With a 5 μL injection of the 25 nM sample, the data in Figure 3-12 represent the consumption of 125 femtomoles of each peptide. The sensitivity of cLC/MS analyses is discussed in further detail later in this chapter.

Inspection of the data in Figure 3-12 reveals that the two early eluting peptides (≤ 20 min.) are again significantly band broadened. This result was previously observed when TFA was employed as the mobile phase modifier (Figure 3-9), yet in this case, formic acid was the modifier. Owing to their polar nature and smaller size, these early eluting peptides are likely migrating from the head of the capillary column during the sample loading and preconcentration period prior to initiation of the LC gradient. Hence, this longitudinal band broadening is manifested in the cLC/MS data as significantly wider peaks for these peptides.

Assessment of the Gradient Elution Problem

Systematic examination of the cLC/MS data from the standard peptide mixture from a qualitative standpoint indicates that too shallow of a gradient ramp causes longitudinal band broadening to the extent that analytes may no longer be detected. A more quantitative approach to assess these results is to apply the linear solvent strength theory.¹⁸³ Specifically, the gradient steepness parameter, b , is used to determine the optimal gradient and is related to the chromatographic capacity factor, k'' ,¹⁸⁴ by the equation¹⁸⁵

$$b = \frac{1}{1.15k''}. \quad (3-1)$$

The generally accepted range of optimal k'' values for isocratic separations is between 1 and 10;¹ however, for gradient separations where the linear velocity and column pressure vary, the suggested optimal capacity factor is 3-6.¹⁸³ From Equation 3-1, this yields b values of 0.290 to 0.145. Calculation of the gradient steepness parameter is also achieved with the following expression

$$b = \frac{(\Delta\Phi)SV_0}{t_G F} \quad (3-2)$$

where $\Delta\Phi$ is the change in the organic composition, S is the organic solvent strength value, V_0 is the void volume of the capillary column, t_G is the time of the gradient ramp, and F is the volumetric flow rate. Taking into account that methanol was the organic solvent for all cLC/MS experiments, the solvent strength value is 3.0.¹⁸³ Also, the length of the chromatographic bed for the 50 μm I.D. columns was approximately 10 cm, equating to a void volume of 140 nL, assuming a column porosity of 0.7.¹⁸⁵ Hence, the

calculated b values for the 1% (Figure 3-8), 2% (Figure 3-7) and 4% per minute (Figure 3-12) gradient ramps from the presented cLC/MS results are 0.038, 0.076, and 0.116, respectively. Notice the gradient steepness parameters are not integer multiples of another due to the experimental variations in the gradient method. Specifically, the change in organic composition and the volumetric flow rate were lower for the cLC/MS acquisitions using 1% and 2% per minute gradients. From these b values it is evident that the 1% and 2% per minute gradients are significantly outside the optimal range for the gradient steepness parameter. Furthermore, the $b = 0.116$ for the gradient ramp of 4% per minute is suboptimal, even though the calculated capacity factor, k'' , for this method is 7.5.

All the cLC/MS and cLC/MS/MS experiments conducted hereafter employed the 4% per minute gradient ramp. While the results were deemed satisfactory from a mass spectrometrists' point of view, it is evident, in retrospect, that the chromatographic integrity of these data is marginal. Nevertheless, the data in the remainder of this dissertation are disclosed in a manner that reflects the successes of this research project due to the incorporation of capillary LC with the developed QITMS.

Analysis of Tryptic Digest

To further examine the ability of the cLC/MS instrument, a tryptic digest of the protein triose phosphate isomerase (TPI), graciously provided by the University of Florida ICBR Protein Core, was analyzed. Briefly, trypsin is an endoprotease that hydrolyzes proteins specifically on the C-terminal side of either lysine or arginine. Hence, a basic site is located on each end of a tryptic peptide (i.e., the Lys or Arg side chain on the C-terminus and the free amine on the N-terminus), which often produces

doubly charged ions by ESI-MS. The tryptic digestion was conducted on the TPI protein ($M_r = 26,626$ u) without any prior modifications (e.g., alkylation of cystine residues). A sample of the digest was diluted to a concentration of 20 nM and a 5 μ L injection was analyzed by cLC/MS (Figure 3-13). The eight observed TPI tryptic peptides are presented in Figure 3-13, which shows baseline chromatographic separation of these peptides by using a 4% per minute gradient.

The peptide mass map generated from these data was submitted to a protein database (ProteinProspector, UCSF). The returned results revealed that the highest probability protein was rabbit muscle triose phosphate isomerase and that the identified tryptic peptides represented 39% amino acid coverage of the protein. The MALDI-MS data of the TPI digest, conducted by the ICBR Protein Core staff, obtained 68% coverage of the protein. Comparison of those data with the cLC/MS results showed that cLC/MS did not observe several of the high-mass tryptic peptides (>2000 u). One possible explanation for not identifying the larger peptides is that these tryptic peptides might not have eluted from the capillary column, even at 90% methanol, due to the increased hydrophobicity of such peptides. Using a more hydrophobic solvent, for example acetonitrile, may have allowed identification of the larger TPI tryptic peptides. Another possibility is that these hydrophobic peptides may have gone undetected due to extreme band broadening in light of the marginally optimized gradient method employed for this analysis (see previous section). Regardless, observation of large (>2000 u) peptides by cLC/MS was not a major goal of this dissertation project and additional experiments on the TPI digest were not pursued.

Sensitivity of cLC/MS

Incorporation of capillary liquid chromatography with the reformed QITMS was aimed at improving the selectivity and sensitivity of analyses, particularly for biological analytes. This has been established above and is further demonstrated with the cLC/MS analysis of the standard peptide mixture (Table 3-1) at a concentration of 2.5 nM spiked with 0.2 ppb spiperone (Figure 3-14). The 5 μ L injection of this solution corresponds to consumption of 12.5 femtomoles of each peptide and 1 picogram of spiperone. Peak widths of the eluting compounds are approximately 0.25 minutes, which, when the flow rate of 144 nL/min. is taken into account, equates to an averaged concentration of 347 nM for the eluting peptides. Hence, the concentration effect due the cLC column is on the order of 140-fold.

Six of the seven peptides in the sample are clearly observed above the chemical noise in the ion chromatograms (Figure 3-14). However, when compared to the base peak chromatogram, which indicates the intensity of the most abundant ion in the mass spectrum, most of the peptide ions' signals do not rise above the level of the background. This fact is demonstrated in Figure 3-15, which displays the base peak chromatogram and ion chromatograms for angiotensin I (m/z 433.1), SDPFLLRFamide (m/z 441.0) and ASGDPNFLRFamide (m/z 562.1). Notice that the $(M+3H)^{+3}$ ion of angiotensin I and the $(M+2H)^{+2}$ ion of ASGDPNFLRFamide do not reach sufficient signal strength to be observed in the base peak chromatogram. For unknown analytes, it would be difficult to identify such compounds from the base peak chromatogram. Furthermore, current mass spectrometers that use "data-dependent" methods to switch the instrument operation from

single-stage MS to MS/MS mode when an ion signal crosses the background threshold would be ineffective under these conditions.

The concentration effect of capillary LC in conjunction with μ ESI-MS has permitted the detection of angiotensin I in single-stage MS mode at a concentration of 2.5 nM, improved from 10 nM for direct infusion of angiotensin I. It was anticipated that the concentration detection limits would be enhanced to a greater degree with cLC/MS, especially in light of the fact that eluting analytes were preconcentrated approximately 100 times their initial sample concentration. A likely reason for not observing larger improvements in the concentration detection limits is due to a higher level of chemical background when packed capillary columns were connected to the μ ESI-MS instrument. This higher chemical background observed with packed f.s. capillaries as opposed to "open" fused silica capillaries (data not shown) may be attributed to the continuous elution of the hydrolyzed stationary phase or other contaminants from the silica particles. Nevertheless, the modest gains in concentration sensitivity demonstrated by cLC/MS are further advanced by the use of MS/MS detection coupled with cLC separations.

Dual MS/MS Monitoring

As discussed in the previous chapter, tandem mass spectrometry affords the ability to obtain structurally informative fragmentation spectra and concurrently improve the S/N ratio of the resultant data. This advantage is widely used in the pharmacological, toxicological and biological sciences for qualitative and quantitative analyses via LC/MS/MS. For known compounds or their analogs, such analyses are relatively straightforward by developing methodology using standards on-hand. For previously unidentified analytes, this task becomes significantly more difficult. One of the common

mass spectral means by which unknowns are presently identified is the use of “data-dependent” scanning techniques for chromatographically separated compounds. Basically, this entails the switching from single-stage MS scanning to MS/MS scanning when an ion produced from an eluting compound is observed above the background noise level. After acquiring a few spectra in the MS/MS mode, the instrument reverts back to full-scan MS mode until another ion exceeds the user preset threshold level whereby the cycle is repeated. This is a highly effective method of obtaining the maximum amount of data on the minimum amount of defined sample parameters (e.g., concentrations of compounds, masses of analytes). However, the major problem with data-dependent scanning is that compounds that do not produce signals above the background noise level go undetected by this mode of operation.

From the cLC/MS results obtained with the 2.5 nM peptide mixture, it was evident that a data-dependent scanning routine, which first needed to be implemented on the PHOENIX instrument, would likely be unproductive for the eventual analysis of the neuropeptide samples due to their unknown concentrations and high complexity. Therefore, an alternate means of obtaining cLC/MS/MS data was explored: dual MS/MS monitoring. Dual MS/MS monitoring operates by alternating cycles of collision-induced dissociation (CID) of two separate m/z ions that are designated by the user, generating two product ion spectra. In other words, a batch of ions is injected into the QIT followed by isolation and excitation of the first specified ion and subsequent ejection of the fragment ions; this entire process is then repeated for the second indicated m/z ion. Unlike data-dependent scanning, dual MS/MS monitoring continually operates in MS/MS mode. While the Visual Basic code had to be written to activate this method of scanning

on the PHOENIX instrument, the groundwork for dual MS/MS scanning had already been incorporated into the C++ code of the advanced GCQ software.

A demonstration of the cLC/MS/MS results with dual MS/MS monitoring is given in Figure 3-16. A 5 μ L aliquot of the aforementioned peptide standard mixture (see Table 3-1) at a concentration of 250 pM was injected and analyzed by cLC/MS/MS by monitoring the ions at m/z 433.0 and 440.8. These ions correspond to the $(M+3H)^{+3}$ and $(M+2H)^{+2}$ of angiotensin I and SDPFLRFamide, respectively. Ion chromatograms drawn for the sequence specific product ions of angiotensin I at m/z 513.2 and 647.3 and the product ions for SDPFLRFamide at m/z 339.8 and 581.3 clearly mark the elution of these peptides at a concentration ten times lower than that in prior cLC/MS analyses (see Figure 3-14). The product ion spectra for the chromatographic peaks at 21.76 min. (Figure 3-17A) and 21.98 min. (Figure 3-17B) are consistent with angiotensin I and SDPFLRFamide, respectively.

Testing the ultimate detection limits of the instrument via cLC/MS/MS, a 5 μ L injection of the peptide standard mixture (Table 3-1) at 10 pM was analyzed. Dual MS/MS monitoring of m/z 433.0 and 561.9 generated the data displayed in Figure 3-18. From the TIC there are three chromatographic peaks observed. The MS/MS data for the peaks at 10.13 min. and 10.52 min. are given in Figures 3-19A and B, where the labeled product ions are consistent with the sequences of angiotensin I and ASGDPNFLRFamide, respectively. Differences in the elution times for angiotensin I between Figures 3-16 and 3-18 are due to a delay in the start of data acquisition employed for Figure 3-18. This acquisition delay was instituted for dual MS/MS monitoring to avoid the collection of unnecessary data, since there was an approximately

10 minute period between the initiation of the gradient at the LC pump and the time that the gradient composition reached the capillary column. These cLC/MS/MS data in Figures 3-18 and 3-19 represent the consumption of 50 attomoles of each peptide. More importantly, angiotensin I is detected at a concentration two orders of magnitude lower than either cLC/MS analyses or μ ESI-MS/MS acquisitions by direct infusion.

The coupling of capillary liquid chromatography with mass spectrometry and MS/MS has been demonstrated here to afford a high degree of concentration and mass sensitivity as well as analyte selectivity. This cLC/MS/MS setup described herein provides sample preconcentration on the order of 100-fold, can identify biological compounds based on their mass-to-charge, and gathers structurally informative data on analytes. Furthermore, the ability to observe peptides at concentrations of 10 picomolar and at mass amounts of 50 attomoles approached that of recent reports in the current mass spectrometric literature.^{141,142,176} All of the aforementioned capabilities are required to achieve success in the application of the cLC/ μ ESI-QITMS toward the identification of neuropeptides within the complex matrix of the *Caenorhabditis elegans* samples.

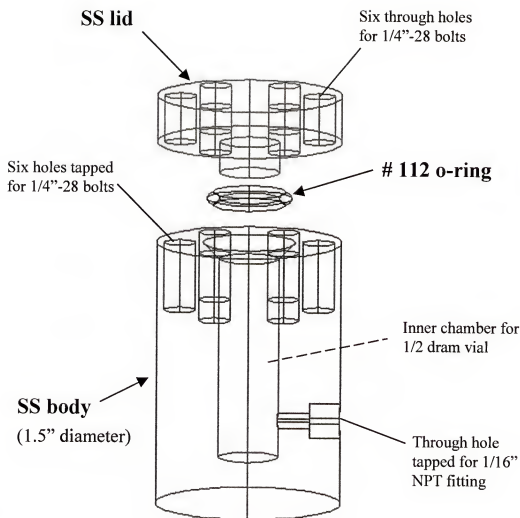


Figure 3-1: 3D wireframe drawing of the high pressure SS bomb. This apparatus was used for packing capillary LC columns and for loading samples onto the capillary column in early cLC/MS experiments. A Valco SS union, omitted from the drawing, was welded into the center of the lid to couple the fused silica capillary tubing to the inner chamber of the SS bomb.

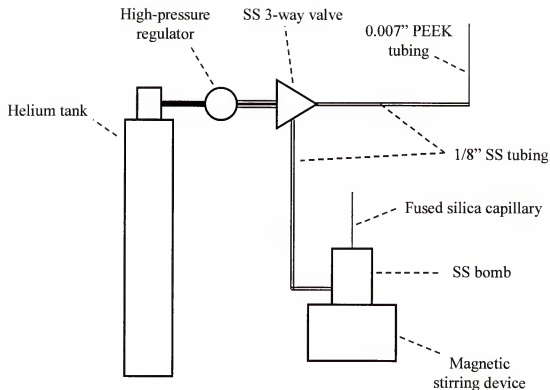


Figure 3-2: Block diagram of capillary column fabrication setup. Packing the fused silica capillary is achieved by switching the three-way valve to allow helium to flow from the tank into the SS bomb, which contains a slurry of the stationary phase. The SS bomb is depressurized by switching the directional flow from the bomb to the atmosphere via the PEEK restrictor tubing. The valve's third position prohibits fluid flow in any direction.

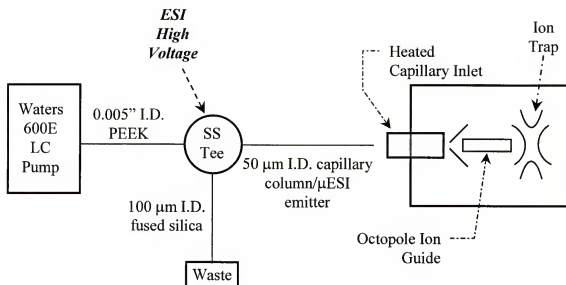


Figure 3-3: Block diagram of the cLC/MS setup. The LC effluent is split at a ratio of approximately 4000:1, providing 100-200 nL/min. to the cLC column/ μ ESI emitter.

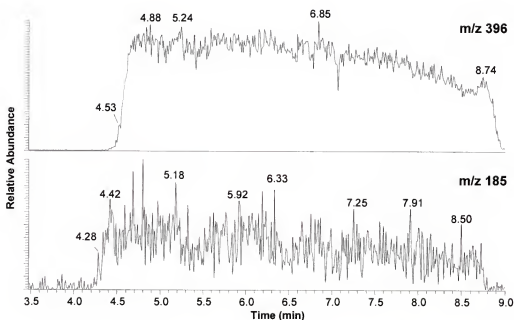


Figure 3-4: Ion chromatograms for a 2.3 mL loop injection of spiperone at 0.28 minutes. The $(M+H)^+$ ion at m/z 396 indicates that spiperone is retained slightly by the C_{18} stationary phase when injected into a stream of 90% methanol as evidenced by the observation of the m/z 185 contaminant ion eluting just before spiperone. The calculated flow rate for the 50 μ m I.D. capillary column is 175 nL/min. (see text for details).

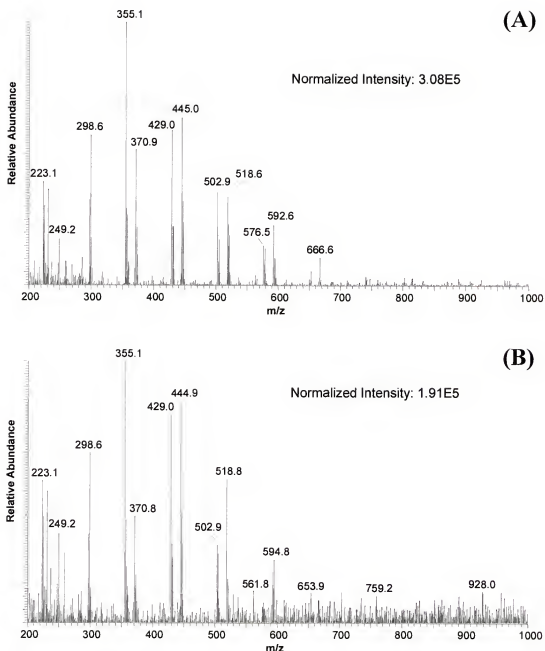


Figure 3-5: μ ESI-MS data of 25% aqueous methanol at varying μ ESI emitter/heated capillary distances. (A) An emitter/capillary distance of approximately 0.75 mm yields a discharge-like mass spectrum as evidenced by the repeating cluster ions (e.g., 355^+ , 429^+ , 503^+) and elevated ion intensity. (B) At the greater distance of about 1.75 mm, the mass spectrum displays a reduction of the repeating cluster ions ($\Delta m=74$ u) and the observation of ubiquitous chemical noise across the entire m/z range.

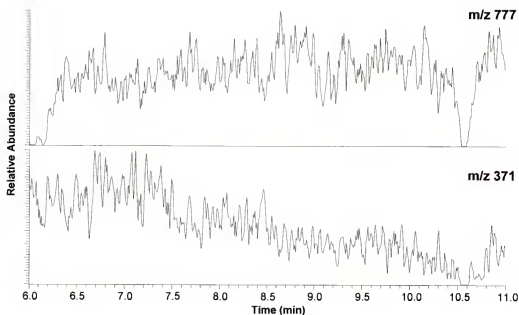


Figure 3-6: Ion chromatograms displaying ion signals as a function of the ESI high voltage. As the ESI high voltage is decreased from +4.3 kV (at 7.0 min.) to +1.6 kV (at 10.5 min.) the intensity of the $(M+H)^+$ of neurotensin 1-6 at m/z 777 remains relatively constant while the background ion at m/z 371 steadily decreases across this range. The optimal signal-to-noise ratio is obtained when the ESI high voltage is about 200-500 V above the ESI onset voltage.

Table 3-1: List of peptides included in the standard mixture for cLC/MS analyses.

Compound	Monoisotopic mass (u)	Observed ions (m/z)
Neurotensin (1-6)	776.4	777.5
GFGDEMamide	653.2	654.3
Angiotensin I	1295.7	433.0, 649.1
SDPFLLRFamide	879.5	441.0, 880.5
ASGDPNLLRFamide	1121.6	561.9, 1122.6
[Lys ³]-Bombesin	1590.8	796.5, 1591.9
Eledoisin	1187.6	594.9, 1188.7

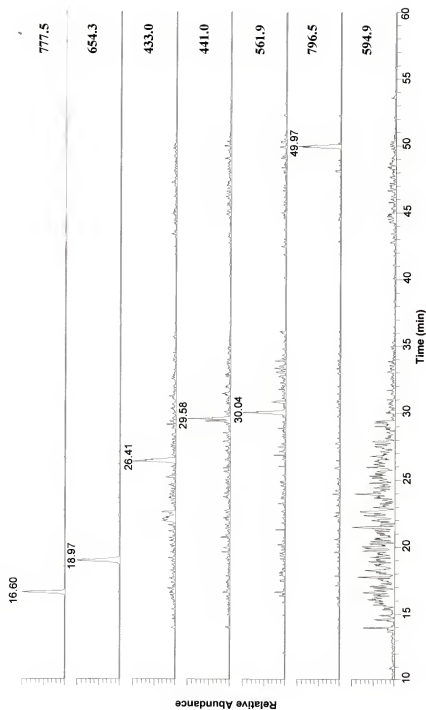


Figure 3-7: cLC/MS of standard peptide mixture using a 2% per min. gradient. The m/z designations for the individual peptide ion chromatograms are given on the right of each trace. From the MS data, the component eluting at 49.97 minutes is an unidentified contaminant and not [Lys⁵]-bombesin.

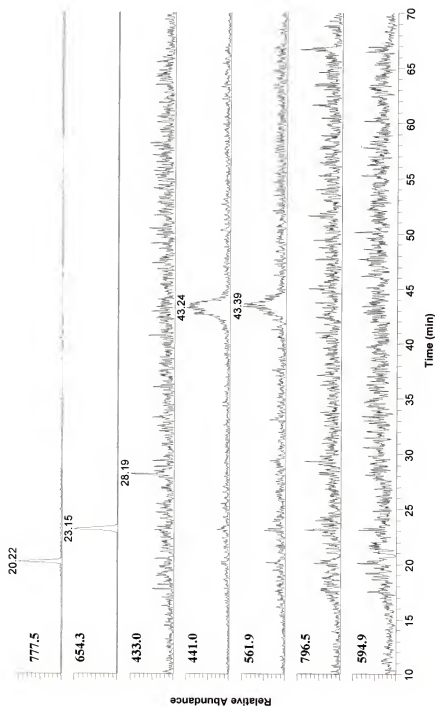


Figure 3-8: cLC/MS of standard peptide mixture using a 1% per min. gradient. The m/z designations for the individual peptide ion chromatograms are given on the left of each trace.

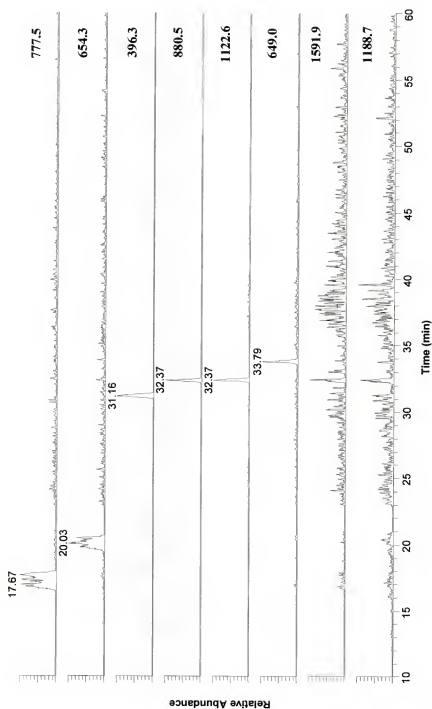


Figure 3-9: cLC/MS of the standard peptide mixture with 20 pbp spiperone using TFA as the mobile phase modifier. A 2% per minute gradient ramp was employed during this acquisition. The m/z designations for the ion chromatograms are given on the right of each trace.

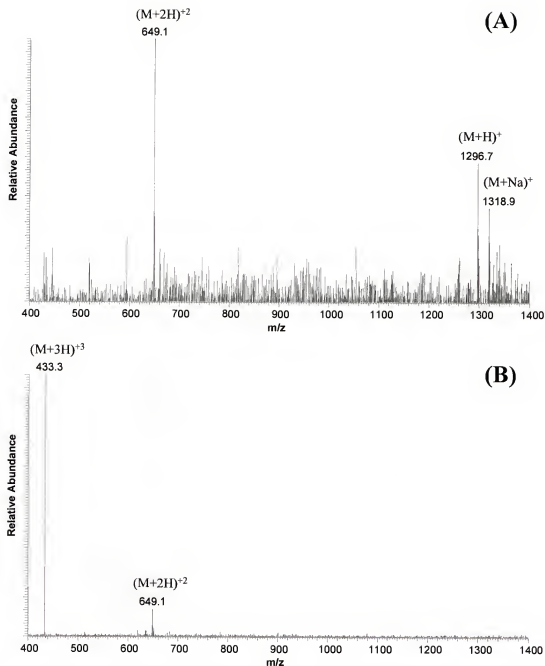


Figure 3-10: Mass spectra of angiotensin I from cLC/MS acquisitions. (A) Observed μ ESI-MS data of 25 nM angiotensin I using 5 mM TFA as the mobile phase modifier. (B) The μ ESI-MS data of 5 nM angiotensin I observed when 0.15% formic acid was employed as the mobile phase modifier.

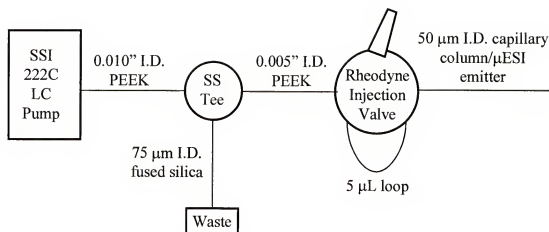


Figure 3-11: Block diagram of the off-line injection apparatus. The mobile phase provided by the LC pump was equivalent to the A mobile phase of the gradient LC pump on the cLC/MS setup. Samples were injected onto the cLC column/ μ ESI emitter at a flow rate of approximately 1 μ L/min.

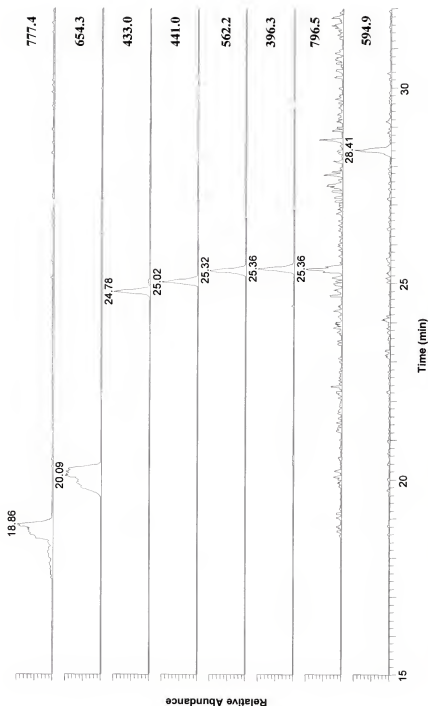


Figure 3-12: cLC/MS of the 25 nM standard peptide mixture with 2 ppb spiperone using a 4% per minute gradient. The fixed-volume injection apparatus was employed to deliver 5 μ L of this sample to the capillary column prior to the acquisition. The m/z designations for each component are given at the right of each trace.

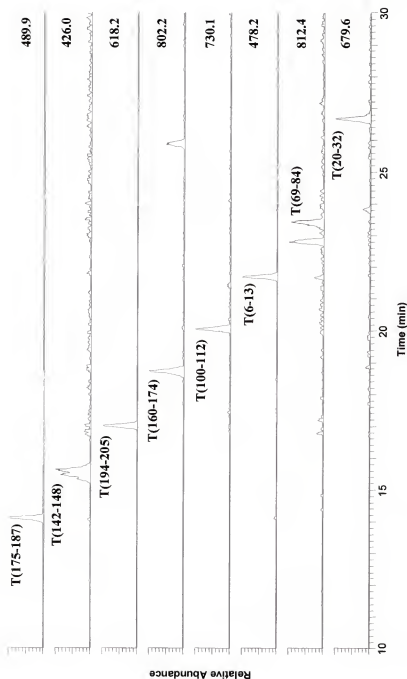


Figure 3-13: cLC/MS of a trypsin digest of 20 nM rabbit triose phosphate isomerase. Individual tryptic peptides are labeled with the amino acid residues in this protein. The m/z designations for each component are given at the right of each trace.

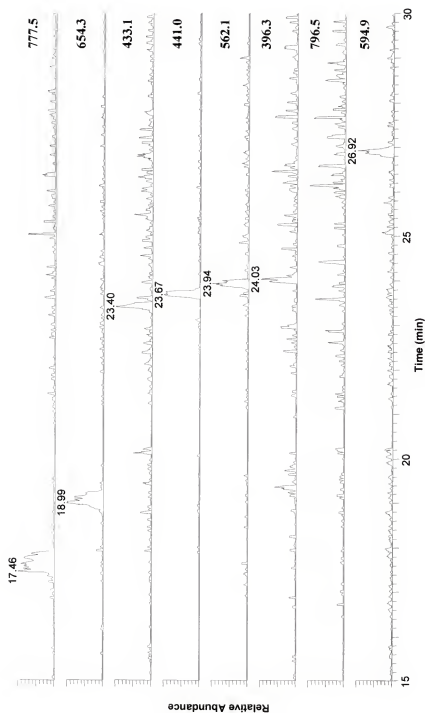


Figure 3-14: cLC/MS of 2.5 nM peptide standard mixture with 0.2 ppb spiperone. A 5 μ L injection of the sample was loaded onto the column prior to the analysis, representing a consumption of 12.5 femtomoles of each peptide and 1 picogram of spiperone. The m/z designations for each component are given at the right of each trace.

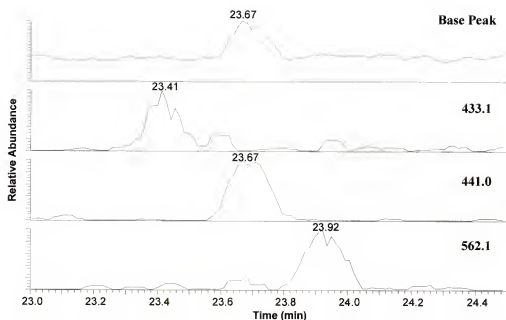


Figure 3-15: Chromatograms from a selected region of 2.5 nM peptide mixture cLC/MS analysis. Comparison of the ion chromatograms to the base peak chromatogram indicates that only the $(M+2H)^{+2}$ of SDPFLRFamide (m/z 441.0) generates an ion signal greater than the level of the maximum background ion current.

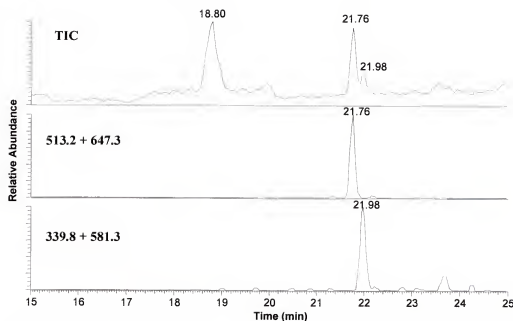


Figure 3-16: Dual MS/MS data for m/z 433.0 and 440.8. Product ions consistent with the peptide sequences of angiotensin I and SDPFLRFamide are listed in the second and third ion chromatograms, respectively. The peak at 18.80 minutes in the total ion current (TIC) is an unidentified contaminant ion with a precursor m/z 433.

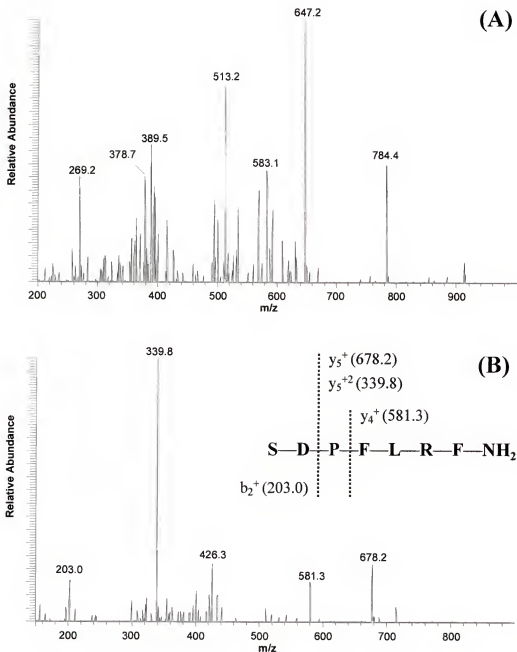


Figure 3-17: Product ion spectra for dual MS/MS monitoring of m/z 433.0 and 440.8. (A) MS/MS data for m/z 433.0 at 21.76 min. in Figure 3-16. This product ion spectrum is consistent with angiotensin I (compare to Figure 2-30). (B) MS/MS data for m/z 440.8 at 21.98 min. in Figure 3-16. Inset displays the fragment ion information for the SDPFLRFamide peptide.

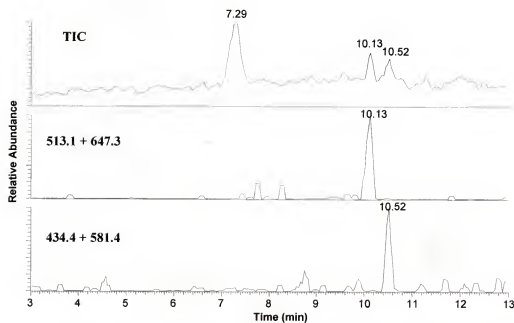


Figure 3-18: Dual MS/MS data for m/z 433.0 and 561.9 from the 10 pM standard peptide mixture. The product ion chromatograms are consistent for the peptide sequences of angiotensin I (second trace) and ASGDPNFLRFamide (third trace). With a 5 μ L sample injection, these data represent a consumption of 50 attomoles of each peptide.

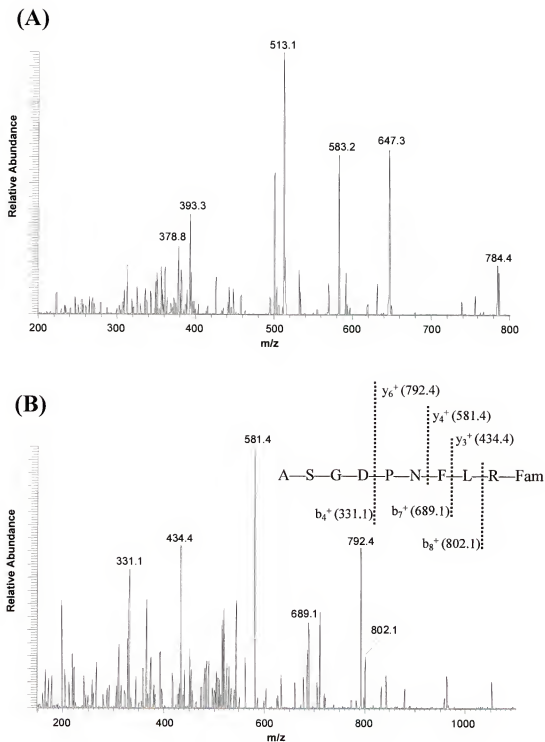


Figure 3-19: Product ion spectra for dual MS/MS monitoring of m/z 433.0 and 561.9. (A) MS/MS data from peak at 10.13 min. in Figure 3-18, which is consistent with angiotensin I. (B) MS/MS data from peak at 10.52 min. in Figure 3-18. Inset gives the fragmentation scheme for the ASGDPNFLRFamide peptide.

CHAPTER 4

INVESTIGATION OF NEUROPEPTIDES IN CAENORHABDITIS ELEGANS

A class of neuropeptides called FMRFamide-like peptides, or FLPs, has received significant attention since the isolation of FMRFamide from the ganglia of the clam *Macrocallista nimbosa* in 1977.¹⁸⁶ In the years that have followed, FLPs have been identified in a wide range of invertebrates and vertebrates, including humans.¹⁸⁷ The physiological activity and function of FLPs are also widely varied and include activation or suppression of muscle contractions in targeted areas (e.g., heart,^{188,189} oviduct¹⁹⁰), a potential antidiuretic,¹⁹¹ and regulation of pain receptors.^{192,193}

The commonly accepted definition for this class of peptides is that the C-terminus ends with the sequence Arg-Phe-NH₂. The sequence length of FLPs generally varies from four to twenty amino acids. For example, a list of the putative FLPs in the nematode *Caenorhabditis elegans* is given in Table 4-1.^{194,195} Most of these potentially expressed FMRFamide-like peptides were determined from the genome of *C. elegans*. With the completion of the genome sequencing in 1998,¹⁹⁶ *C. elegans* is an ideal organism with which to investigate FLPs. There have been 20 genes identified that potentially express 56 FLPs. Some of these FLPs have been previously confirmed by other means^{194,195} and those peptide sequences are italicized in Table 4-1.

The application goal of this dissertation project is to identify as many of the putative FLPs in *C. elegans* with as little sample preparation as possible. Accurately identifying the FLPs at trace levels would afford the opportunity to study the

neurobiology of *C. elegans*. For example, the presence or absence of certain FLPs in the developmental stages of this organism may lend key information as to the roles played by these FMRFamide-like peptides. The cLC/MS/MS instrumentation and methodology described in the previous chapters is applied here to investigate *C. elegans* extracts for the presence of FLPs.

cLC/MS of *C. elegans* Extracts

Since the Yost lab is not equipped to work with biological organisms, the *C. elegans* samples were prepared by the Dr. Arthur Edison group at the University of Florida Brain Institute. The procedure for preparing the extracts is given in the outline below.

- 1) Place 10-20 mg wild-type *C. elegans* into 250 μ L acetone
- 2) Freeze/thaw cycles (4-5 repeats) of the acetone suspension in liquid N₂; homogenize during the thaw cycle with fine glass rod
- 3) Centrifuge at 16,000 g for 15 minutes
- 4) Decant supernatant
- 5) Evaporate to dryness under N₂ stream
- 6) Remove lipids via liquid extraction with 1:1 (%v/v) ethyl acetate:diethyl ether
- 7) Reconstitute sample with deionized water

This method of sample preparation provided approximately 25 μ L of the *C. elegans* extract, which was stored at -4° C until analysis.

Prior to the cLC/MS data acquisition, the *C. elegans* sample was diluted by adding 2 μL of the aqueous extract to 4 μL of 5% methanol plus 0.15% formic acid containing angiotensin I at a concentration of 25 nM. By spiking the *C. elegans* extract with angiotensin I, the integrity of the cLC/MS data could be confirmed, and the sensitivity of the system could be ascertained for a complex sample in single-stage MS mode. A 5 μL injection of the 1:3 dilution *C. elegans* extract was realized using the off-line injector as described in Chapter 3.

The chromatographic conditions employed for the cLC/MS analysis are similar to those discussed in the previous chapter. Briefly, the capillary column is 50 μm I.D. with a packing bed length of approximately 10 cm. The binary mobile phase system consisted of 5% methanol (A) and 90% methanol (B), both of which contained 0.15% formic acid as a modifier. A 4% per minute linear gradient from 1% B to 99% B was used to elute the components from the cLC column. For the cLC/MS analysis of the *C. elegans* extract, the calculated column flow rate was 188 nL/min.

Figure 4-1 displays the cLC/MS results for the wild-type *C. elegans* extract. Observation of angiotensin I is demonstrated by the 20.25 min. peak on the m/z 433.3 ion chromatogram. With a net concentration of 16.7 nM, this represents the consumption of 83.3 femtomoles of angiotensin I. Inspection of the TIC and base peak chromatograms reveals that the *C. elegans* extract is highly complex. Analysis of the significant peaks in these cLC/MS data did not correlate to any of the FLPs listed in Table 4-1. Further examination of the cLC/MS results by selected ion chromatograms of the doubly charged ions (since FLPs have a basic site on the N- and C-terminal regions) of the previously identified FLPs (see Table 4-1) also failed to confirm the presence these peptides. Since

angiotensin I was observed in this sample at 83.3 femtomoles by cLC/MS, it was concluded that the mass loading of the FLPs was lower than 10 femtomoles. Therefore, escalation of analyses was required to achieve improved sensitivity. As previously demonstrated, this is accomplished by cLC/MS/MS.

cLC/MS/MS of *C. elegans* Extracts

The advantage of working with an organism with its genome completely mapped is that the amino acid sequences and peptide masses of the target analytes are known. In the case of these *C. elegans* extracts, the information in Table 4-1 is of paramount importance, since the charge state generated by ESI can be inferred from the peptide sequence. Due to the fact that the targeted FLPs could not be distinguished from the chemical noise in the cLC/MS data, dual MS/MS monitoring was employed using the m/z values of the doubly protonated FLPs listed in Table 4-1. By adding another dimension of analysis in the examination of the *C. elegans* samples, a greater selectivity is provided to help identify the putative FLPs in the complex matrix.

Due to the limitation of the dual MS/MS monitoring routine to examine only two precursor ion m/z values during a given LC acquisition, the FLPs that had been previously identified (Table 4-1) were the peptides initially investigated by cLC/MS/MS. Furthermore, initial cLC/MS/MS analyses used samples that were spiked with angiotensin I to confirm the accuracy of the cLC/MS/MS results. An example of the acquired data using a 5 μ L injection of a 1:3 dilution of the *C. elegans* extract spiked with 67 pM angiotensin I is shown in Figure 4-2. For this analysis, the precursor ions 433.0 and 561.9 were sequentially subjected to MS/MS during the chromatographic run, corresponding to the $(M+3H)^{+3}$ for angiotensin I and to the $(M+2H)^{+2}$ for

ASGDPNFLRFamide, respectively. Ion chromatograms for the product ions of angiotensin I (i.e., m/z 513.3, 647.3) and ASGDPNFLRFamide (i.e., m/z 792.5, 802.3, 952.3) confirm the identification of these peptides (Figure 4-2). The MS/MS data for the peaks at 11.98 and 12.29 minutes (elution times uncorrected for 7.5 min. acquisition delay) are given in Figures 4-3A and B. An interesting aspect of the product ion spectrum in Figure 4-3B is that there is a major ion at m/z 486.1 which is not consistent with a possible fragment ion of ASGDPNFLRFamide. The ion chromatogram for m/z 486.1 (Figure 4-2) indicates from its peak shape that it is another compound that happens to have a precursor m/z of 561 or 562 (unknown due to the ion isolation width used). Inspection of the TIC of Figure 4-2 reveals the presence of numerous components within the *C. elegans* extract yielding m/z of either 433.0 ± 1.0 or 561.9 ± 1.0 , indicating the immense complexity of this sample.

Additional cLC/MS/MS analyses monitoring mass-to-charges consistent with the $(M+2H)^{+2}$ ions of FMRFamide-like peptides from the *flp-1* gene did not confirm the presence of the selected FLPs within the *C. elegans* extract. As in Figure 4-2, the TICs of the cLC/MS/MS acquisitions indicated several compounds at the monitored masses, yet none of the product ion spectra were consistent with the sequences of the FLPs. In the interest of scientific curiosity, the MS/MS data of some of these peaks were manually sequenced and the partial sequence tag was entered into a *C. elegans* database. The obtained results were inconclusive, often receiving tens of possible proteins from which these sequences could originate and none that correlated to a FLP precursor protein.

To reaffirm the identification of the one FLP, examination of the *C. elegans* extract via cLC/MS/MS was repeated several days later by monitoring m/z 451.5 and

561.9, consistent with the $(M+2H)^{+2}$ ions of KSAYMRamide (*flp-6* gene) and ASGDPNFLRamide. Although the ion chromatogram for the ASGDPNFLRamide product ions in Figure 4-4 (i.e., second trace) is significantly more complex than the previous results (Figure 4-2), the compound-specific peak can be found at 13.78 minutes (uncorrected for acquisition delay of 7.0 min.). The resultant MS/MS data for m/z 561.9 at 13.78 minutes are displayed in Figure 4-5. While the product ion spectrum in Figure 4-5 appears to have a great deal of background noise, the three previously indicated sequence-specific ions (i.e., 792.4, 802.3, 959.3) for ASGDPNFLRamide are observed. However, the inability to confirm the previously identified KSAYMRamide peptide¹⁹⁴ during this cLC/MS/MS analysis coupled with the poor signal-to-noise ratios of the data in Figures 4-4 and 4-5 calls into question the validity of the conclusion that the FLP ASGDPNFLRamide was positively identified in Figure 4-2.

A possible explanation for observing the ASGDPNFLRamide peptide and not any of the other previously identified FLPs is that the peptide standard mixture (Table 3-1) used to optimize the cLC/MS(/MS) parameters contained ASGDPNFLRamide. While considerable efforts were made to rinse the various components of the cLC/MS system (e.g., syringe, injection valve), trace amounts of this peptide may have remained in one of these items and could have been inadvertently injected onto the cLC column during the analysis of the *C. elegans* extracts. Unfortunately, a blank cLC/MS/MS analysis on the m/z 561.9 was not acquired, and by the time this experimental factor had been realized the entire *C. elegans* extract had been consumed. Therefore, the identification of one of the FLPs in the wild-type *C. elegans* is highly questionable at best.

Based on the results presented here, it is hypothesized that the inability to adequately identify the FLPs from the original extracts may be due to several possibilities, including protease hydrolysis of the FLPs during the extraction procedure, an insufficient amount of the peptides in the sample and sample handling losses during the various steps prior to cLC/MS/MS analyses. It was hoped to continue the investigation for FLPs within *C. elegans* by obtaining new samples with an improved extraction procedure. However, the Edison group has shifted their research on FLPs to another organism and new *C. elegans* extracts were not forthcoming.

While the observation of the FLP ASGDPNFLRFamide in the *C. elegans* extract is in dispute, the cLC/MS/MS methodology developed to identify these peptides is sound. For example, the standard peptide angiotensin I was observed within this complex sample matrix at the level of 83 femtomoles in single-stage MS mode and identified via cLC/MS/MS by loading 335 attomoles of angiotensin I on column. Therefore, the groundwork for further investigation of FLPs in *C. elegans* or in another organism is in place for future endeavors of this project.

Table 4-1: List of the putative FMRFamide-like peptides in *C. elegans*. The peptides in italics are FLPs that have been previously identified by various means.

Gene	Sequence	Mass (u)
flp-1	<i>SADPNFLRFa</i>	1064.529
	<i>SQPNFLRFa</i>	1006.524
	<i>ASGDPNFLRFa</i>	1121.551
	<i>SDPNFLRFa</i>	993.492
	<i>AAADPNFLRFa</i>	1119.571
	<i>PNFLRFa</i>	791.443
	<i>AGSDPNFLRFa</i>	1121.551
flp-2	<i>SPREPIRFa</i>	999.550
	<i>LRGEPIRFa</i>	985.571
flp-3	<i>SPLGTMRFa</i>	906.464
	<i>TPLGTMRFa</i>	920.479
	<i>EAEELPGTMRFa</i>	1277.597
	<i>NPLGTMRFa</i>	933.475
	<i>ASEDALFGTMRFa</i>	1342.623
	<i>EDGNAPFGTMRFa</i>	1339.587
	<i>SAEPFGTMRFa</i>	1140.528
	<i>SADDSAPFGTMRFa</i>	1399.608
	<i>NPRNDTPFGTMRFa</i>	1550.730
flp-4	<i>PTFIRFa</i>	778.438
	<i>ASPSFIRFa</i>	922.491
flp-5	<i>GAKFIRFa</i>	836.491
	<i>AGAKFIRFa</i>	907.528
	<i>APKPKFIRFa</i>	1011.670
flp-6	<i>KSAYMRFa</i>	900.453
flp-7	<i>SPMQRSSMVRFa</i>	1323.643
	<i>TMQRSSMVRFa</i>	1240.606
	<i>SPMERSAMVRFa</i>	1308.632
	<i>SPMDRSKMVRFa</i>	1351.674

Gene	Sequence	Mass (u)
flp-8	<i>KNEFIRFa</i>	951.518
flp-9	<i>KPSFVIRFa</i>	991.586
flp-10	<i>QPKARSGYIRFa</i>	1320.731
flp-11	<i>AMRNALVRFa</i>	1075.596
	<i>ASGGMRNALVRFa</i>	1277.496
	<i>NGAQPPFVRFa</i>	1130.588
flp-12	<i>RNKFEFIRFa</i>	1254.688
flp-13	<i>SDRPTRAMDSPLIRFa</i>	1759.904
	<i>AADGAPLIRFa</i>	1028.566
	<i>APEASPFIRFa</i>	1132.592
	<i>ASPSAPLIRFa</i>	1056.597
	<i>SPSAVPLIRFa</i>	1084.628
	<i>ASSAPLIRFa</i>	959.544
flp-14	<i>KHEYLRFa</i>	990.529
flp-15	<i>GGPQGPLRFa</i>	926.498
	<i>RGPSGLRFa</i>	984.551
flp-16	<i>AQTFVRFa</i>	866.465
	<i>GQTFVRFa</i>	852.450
flp-17	<i>KSAFVRFa</i>	852.486
	<i>KSQIRFa</i>	776.455
flp-18	<i>EMPGVLRFa</i>	946.495
	<i>EIPGVLRFa</i>	928.539
	<i>SEVPGVLRFa</i>	1001.555
	<i>DVPGVLRFa</i>	900.507
	<i>SVPGVLRFa</i>	872.512
flp-19	<i>WANQVRFa</i>	918.471
	<i>ASWASSVRFa</i>	1008.503
flp-20	<i>AMMVRFa</i>	752.372

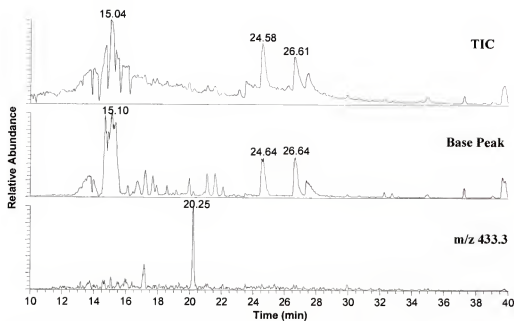


Figure 4-1: Chromatograms for cLC/MS of *C. elegans* extract. The m/z 433.3 ion chromatogram indicates the presence of the $(M+3H)^{+3}$ of angiotensin I, which was spiked into the sample at a level of 83.3 femtomoles. Positive identification of any FLP was not obtained from the ion chromatograms consistent with the FLP masses listed in Table 4-1.

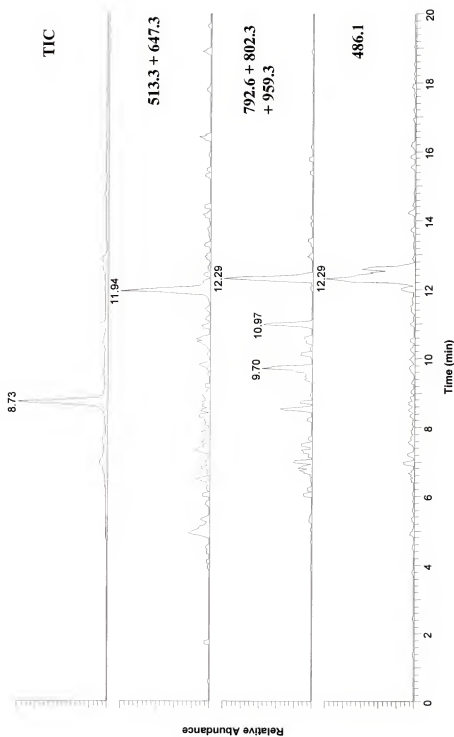


Figure 4-2: cLC/MS/MS data of *C. elegans* extract. Dual MS/MS monitoring was employed in which the ions at m/z 433.0 and 561.9 were subjected to collision-induced dissociation during the LC run. The second and third ion chromatograms are consistent with the product ions for angiotensin I (at 11.94 min.) and ASGDPNPLRFamide (at 12.29 min.). The eluting component at 12.29 with a product ion of 486.1 corresponds to an unidentified compound with a precursor m/z 561.9.

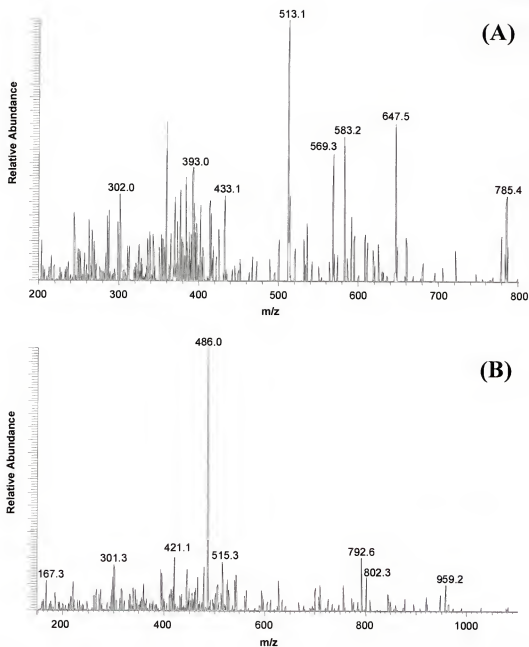


Figure 4-3: Product ion spectra from cLC/MS/MS of *C. elegans* extract. (A) MS/MS data of m/z 433.0 from peak at 11.94 min. in Figure 4-1. Many of the labeled product ions are consistent with the sequence of angiotensin I (compare to Figure 2-30B). (B) MS/MS data from the peak at 12.29 min. resulting from the precursor ion at m/z 561.9. The ions at m/z 792.6, 802.3 and 959.2 are representative of the ASGDPNFLRFamide peptide. The ion at m/z 486.0 is an unknown product ion that is not consistent with the sequence of the observed FLP.

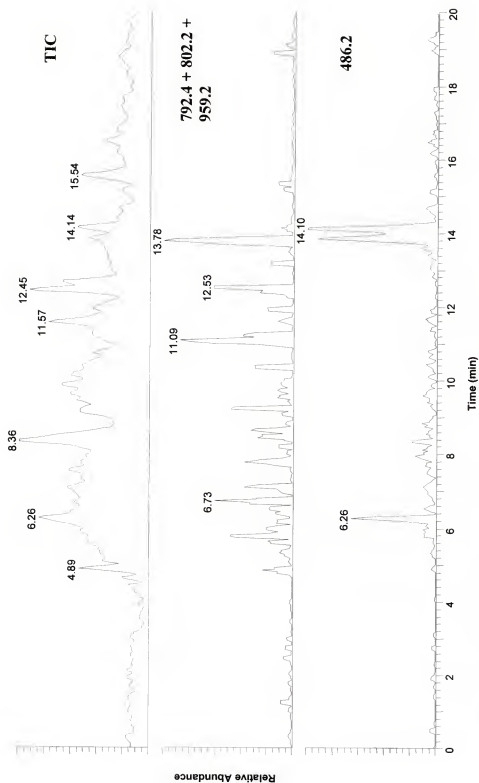


Figure 4-4: cI/C/MS/MS analysis of *C. elegans* extract. Dual MS/MS monitoring was employed for the ions at m/z 451.5 and 561.9 during the LC run. The second ion chromatogram illustrates the product ions consistent with the FLP ASGDPNFLRFamide; the mass spectrum for the peak at 13.78 in displayed in Figure 4-5. The eluting component at 14.10 min. with a precursor ion of 486.2 corresponds to an unidentified compound with a precursor m/z 561.9.

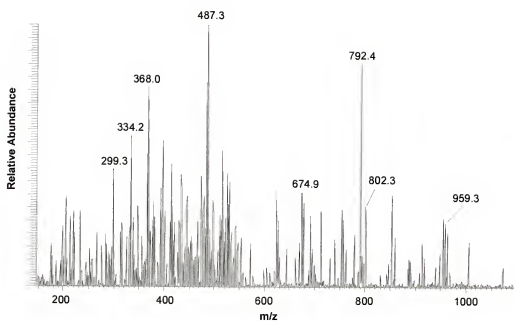


Figure 4-5: Product ion spectrum of m/z 561.9 for peak at 13.78 min. in Figure 4-4. Three of the product ions, 792.4, 802.3 and 959.3, are consistent with the FMRFamide-like peptide, ASGDPNFLRFamide.

CHAPTER 5

CHARACTERIZATION OF THE TOXIC COCAINE METABOLITE NORCOCAINE NITROXIDE BY LC/MS/MS

When cocaine is introduced into a biological system, it is metabolized by two primary pathways.¹⁹⁷ One is the hydrolysis of the benzyl or methyl ester of cocaine by serum and liver esterases to form ecgonine methyl ester or benzoylecgonine, respectively. The other significant metabolic pathway is oxidative. N-demethylation of cocaine by cytochrome P450 enzymes produces the pharmacologically active metabolite norcocaine (NC).¹⁹⁸⁻²⁰⁰ Further N-oxidation of norcocaine yields N-hydroxynorcocaine (NHNC)^{201,202} and norcocaine nitroxide (NCNO)²⁰³⁻²⁰⁵ (Figure 5-1). It has been demonstrated that the N-oxidative metabolites of cocaine, particularly norcocaine nitroxide, are toxic, causing necrosis of liver cells.²⁰⁶ However, since the oxidative biotransformation of cocaine is minor compared to the formation of esterolysis products,²⁰⁷ norcocaine is not one of the key metabolites monitored by analytical methods for detecting cocaine abuse.

Norcocaine nitroxide, a stable organic radical (see Figure 5-1), is currently believed to be the primary metabolite to cause liver damage in mice and humans stemming from prolonged cocaine use.^{197, 206, 208} Although the precise mechanism by which cocaine hepatotoxicity is brought about is not yet known, research has shown that NCNO causes a reduction in mitochondrial respiration,²⁰⁹⁻²¹¹ an increase in lipid peroxidation,^{204,212} and a reduction of liver glutathione levels.²¹³ Additionally, experiments using radiolabeled cocaine resulted in the formation of an irreversibly bound

metabolite to microsomal proteins,²¹⁴ suspected to be NCNO or its oxidized nitrosonium ion analog.

One of the current means of determining the hepatotoxicity of the N-oxidative metabolites of cocaine is to measure the alanine aminotransferase (ALT) activity in serum.²¹⁵ ALT activity has long been recognized in the biological community as a marker for liver cell damage; however, it is an indirect way to measure the hepatotoxicity of an external agent (e.g., drug, virus). A valuable and complementary means to help ascertain the toxicity of N-hydroxynorcocaine and norcocaine nitroxide would be to directly measure the *in vitro* or *in vivo* concentrations of these cocaine metabolites.

Presently, GC/MS is often the analytical method of choice to screen for the use of cocaine.²¹⁶⁻²²⁰ While GC/MS is highly sensitive and selective for detection of cocaine and its metabolites, the extracts from biological matrices must be derivatized prior to analysis due to the polarity and thermal lability of these analytes. To overcome this drawback, LC/MS/MS methods have been recently developed to analyze biological-fluid extracts for the presence of the major cocaine metabolites.²²¹⁻²²⁵ Nevertheless, no mass spectrometry method currently exists to characterize the toxic metabolites NHNC and NCNO from living systems. In fact, to date the only technique used to confirm the existence of norcocaine nitroxide as an N-oxidative metabolite of cocaine has been electron paramagnetic resonance (EPR) spectroscopy.^{203-205,226}

This chapter focuses on the development of an LC/MS/MS method for the purpose of assaying the N-oxidative metabolites of cocaine, with emphasis on the hepatotoxic metabolite norcocaine nitroxide. All mass spectrometry experiments described in this chapter were conducted on the commercially available ThermoFinnigan

LCQ quadrupole ion trap mass spectrometer. The steps involved in characterizing NCNO included the acquisition of the full-scan MS and MS/MS data for this free radical using electrospray ionization, examination of the stability of NCNO in aqueous solutions at different pHs, and optimization of the liquid chromatographic conditions to afford the efficient separation of NC, NHNC and NCNO. It is particularly important to apply the selectivity provided by LC to chromatographically resolve N-hydroxynorcocaine from norcocaine nitroxide, since these compounds differ by only a hydrogen atom (i.e., 1 u). After successfully demonstrating that the N-oxidative metabolites cocaine are readily observed by LC/MS and LC/MS/MS, this method is applied to confirming the production of NHNC and NCNO via *in vitro* incubation of norcocaine and N-hydroxynorcocaine, respectively, with rat liver microsomes.

Mass Spectrometry of Nitroxides

Nitroxides are stable free radicals that have been the focus of much chemical and biological research in recent years due to their interesting properties. For example, nitroxides have been shown to act as good antioxidants in biological systems by oxidizing intracellular radicals, such as lipid peroxides,²²⁷ as well as mimicking the detoxifying enzyme superoxide dismutase (SOD) by reducing $O_2^{\bullet-}$ to H_2O_2 .²²⁸ Also, nitroxides are useful in terminating polymerization reactions by capping the polymer²²⁹ and are employed for monitoring the production of transient radical species by EPR.^{228,230}

The use of mass spectrometry to analyze nitroxides has been limited. At the time of this dissertation only one group had used ESI-MS to examine nitroxides, in which two interesting results were reported.²³¹ First, the ESI-MS sensitivity for the nitroxides they examined was low, typically requiring solutions on the order of 100 ng/ μ L to achieve

satisfactory spectra. Second, Smith et al. observed that ESI-MS of these radical species yielded predominantly M^+ rather than $(M+H)^{+\bullet}$ ions. While the oxidation of organic compounds in positive ion ESI-MS is by no means unprecedented,⁹⁴ it is not commonly observed with analytes containing basic centers or polar sites, where protonation or cationization usually occurs via ESI.

In an effort to reproduce the results of Smith and coworkers,²³¹ a commercially available nitroxide, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl benzoate (or TEMPO benzoate), was obtained and analyzed by ESI-MS. Figure 5-2 shows the resultant positive ion ESI-MS data for a 2 ng/ μ L solution of TEMPO benzoate. As indicated by Smith et al., the ESI-MS sensitivity for the characterization of nitroxides is poor, as revealed by the high background and by the low signal response for TEMPO benzoate (MW=276) at the concentration used. Examination of the diagnostic ions for TEMPO benzoate shows that the molecular ion (M^+) at m/z 276 is indeed produced by the ESI source via one-electron oxidation, yet contrary to the observations of Smith, the base peak generated by ESI is the $(M+H)^{+\bullet}$ (m/z 277) formed by proton transfer. One possible explanation for these conflicting results may be due to the fact that Smith and coworkers used the aprotic solvent acetonitrile as a mobile phase, whereas Figure 5-2 was generated from a methanol/water/acetic acid solution.

A final aspect of the ESI-MS data for TEMPO benzoate (Figure 5-2) is the relative abundance of the m/z 278 ion (48%), which is significantly greater than the calculated ^{13}C isotopic abundance for the $(M+H)^{+\bullet}$ of this compound (17.6%). The elevated abundance of the m/z 278 ion strongly suggests the presence of the hydroxylamine of TEMPO benzoate. The question of whether or not this reduction

product is an impurity of TEMPO benzoate sample or is formed *in situ* at the electrospray emitter (since nitroxides can oxidize Fe^{+2})²³² will be addressed further below in conjunction with the LC/MS/MS characterization of norcocaine nitroxide.

Stability of Norcocaine Nitroxide in Aqueous Solutions

Analytical methods developed to analyze organic compounds by LC/ESI-MS(/MS) commonly use reversed-phase chromatography employing acidified mobile phases. Therefore, prior to developing an LC/MS/MS method for norcocaine nitroxide, a 2 ng/ μL solution of NCNO was prepared in 50% methanol with 0.5% (v/v) acetic acid and examined by direct infusion ESI-MS. Figure 5-3 displays the full-scan ESI-MS data for NCNO (MW = 304) in the acidified aqueous solution analyzed minutes after preparation. Immediately noticeable is that the $(\text{M}+\text{H})^{+*}$ and M^+ ions of NCNO at m/z 305 and m/z 304 are minor compared to the base peak in the mass spectrum at m/z 306. It seemed obvious from these data the ion at m/z 306 was likely attributed mainly to the $(\text{M}+\text{H})^+$ of the reduction product of NCNO, N-hydroxynorcocaine. When the same NCNO sample was stored at room temperature and analyzed 19 hours later, the ESI mass spectrum displays no convincing evidence of the NCNO characteristic ions at m/z 304 and 305 (data not shown). Apparently, norcocaine nitroxide is rapidly reduced to N-hydroxynorcocaine in the presence of acid.

To test this hypothesis, a fresh solution of 5 ng/ μL NCNO in 50% aqueous methanol with a lower concentration of acetic acid (0.1% v/v) was prepared and monitored by ESI-MS over time. Initiating the ESI-MS acquisition at 5 min. after solution preparation, it is clearly evident from Figure 5-4 that NCNO rapidly reacts under acidic conditions. The reconstructed ion chromatograms for the NCNO diagnostic ions at

m/z 305, 322 and 327 (i.e., $(M+H)^{+*}$, $(M+NH_4)^{+*}$, and $(M+Na)^{+*}$ ions, respectively) steadily decrease over the acquisition time of 30 min. with a concomitant increase in m/z 306, the $(M+H)^+$ of NHNC. Based on these results, development of a successful LC/MS/MS method for the assay of NCNO would require the absence of an acid modifier in the mobile phase.

Another important question that needed to be addressed is whether or not NCNO was stable for a long enough period of time in aqueous solutions near neutral pH to allow analyses by LC/MS/MS. Therefore, water/methanol solutions of NCNO were temporally examined both without any additives and with the addition of the neutral buffer ammonium acetate (NH_4OAc), a buffer commonly used with LC/ESI-MS due to its volatility. Figure 5-5 demonstrates that norcocaine nitroxide is indeed stable at room temperature for a period of at least 30 min. as there is no difference within experimental error in the ratio of the abundance of m/z 305, the $(M+H)^{+*}$ of NCNO, to m/z 306, the $(M+H)^+$ of NHNC, in either 50% methanol (Figure 5-5A) or 50% methanol containing 1 mM NH_4OAc (Figure 5-5B). Furthermore, when the NCNO solution in 1 mM NH_4OAc was stored at room temperature for 18 h and reexamined by ESI-MS (Figure 5-6B), there is still no marked difference in the total abundance of the NCNO ions at m/z 305, 322 and 327 relative to the $(M+H)^+$ ions of N-hydroxynorcocaine at m/z 306 when compared to the ESI-MS data acquired at 0.5 h (Figure 5-6A). Note that the relative abundance of m/z 306 to m/z 305 in the ESI mass spectra of Figures 5-6A and B is 34%; therefore, since the theoretical ^{13}C isotopic abundance of the $(M+H)^{+*}$ ion of NCNO is 17.6%, there is a measurable contribution of NHNC at m/z 306 from these data.

Based on these experiments, it is reasonable to conclude that examination of NCNO by LC/MS/MS is possible, since this free radical is stable in aqueous solutions near pH 7 at room temperature for many hours. Additionally, ESI-MS results of neutral aqueous solutions of norcocaine nitroxide have indicated that this N-oxidative metabolite of cocaine is stable for the period of >48 hours when stored at -20°C (data not shown). The next challenge in developing an LC/MS/MS method for the N-oxidative metabolites of cocaine was establishing the chromatographic conditions to afford efficient separation NCNO from its closely related analog NHNC (Figure 5-1) with the restriction of using mobile phases near pH 7.

LC/MS and LC/MS/MS of Norcocaine Nitroxide

As indicated above, the LC/MS(/MS) experiments were conducted on a Thermo Finnigan LCQ optimized for high-flow conditions. Using a Waters 600E LC pump, 2.4 mL loop injections of 500 ng/mL spiperone were delivered to the standard ESI source at a flow rate of 0.2 mL/min. of 50% methanol in order to optimize the LCQ operating parameters. Table 5-1 lists the typical values used for the various LCQ instrument parameters for the LC/MS/MS experiments described herein.

Due to the large swept volume between the Waters 600E mixing chamber and the LC column, a precolumn split was employed to reduce the delay time between the initiation of the gradient and when the solvent front reached the LC column. Based on the column used, the length and I.D. of fused silica tubing from the splitting tee was varied to achieve a split ratio between 3:1 and 5.25:1. The flow rate from the Waters LC pump was set to yield a flow rate of 0.17-0.19 mL/min. to the LC column. Typical flow rates delivered from the LC pump were 0.55-1.3 mL/min., which still resulted in a 5-7

min. delay between the mixer and the column. Finally, to ensure that the entire sample was injected onto the LC column, the precolumn splitting tee was arranged before the injection valve. The injection volume used was 10 μ L unless noted otherwise.

The initial LC/MS/MS experiments employed a 2.1 mm I.D., 100 mm long LC column packed with 5 μ m silica particles modified with a C₁₈ stationary phase (Restek). After development of the LC/MS/MS method for NCNO, NHNC and NC, the silica-based column was replaced with a C₁₈ column using polyvinyl alcohol based copolymer particles (Astek) with similar dimensions (i.e., 2.0 mm I.D., 100 mm long, 5 μ m particles). The rationale for switching to the polymer-based LC column will be described below.

Preliminary attempts to analyze NCNO by LC/MS were conducted with a methanol/water based mobile phase without the use of a modifier. It was anticipated that the lack of a mobile phase buffer would provide sufficient partitioning between the targeted analytes to effect separation and at the same time eliminate any contamination of the ESI source from a build-up of the buffer on the ion optics. The LC/MS results from a 10 μ L injection of 0.5 ng/ μ L NCNO are shown in Figure 5-7. A linear gradient from 0% B to 100% B in 10 min. was employed, where mobile phase A was 5% methanol and mobile phase B was 95% methanol. Based on the ion chromatograms for protonated NCNO at m/z 305 and for protonated NHNC at m/z 306, the observation of N-hydroxynorcocaine at 11.23 min. is obvious, yet there is no clear peak for norcocaine nitroxide. The corresponding mass spectra for the three major peaks yielding ions at m/z 305 at retention times of 6.03, 10.18 and 14.81 min. are displayed in Figure 5-8. The m/z 305 ion for the component eluting at 6.03 min. (Figure 5-8A) is the (M+Na)⁺ for a

polyethylene glycol oligomer, which was later discovered to be a contaminant in the HPLC grade water. Skipping to the peak at 14.81 min., from the ESI-MS data in Figure 5-8C, the ion at m/z 305 is a ^{13}C isotope of the compound having an $(\text{M}+\text{H})^+$ or $(\text{M}+\text{NH}_4)^+$ at m/z 304 and thus cannot be NCNO. As for the peak at 10.18 min. (Figure 5-8B), from these ESI-MS data, the signal-to-noise for the m/z 305 ion is not sufficient to confidently assign this peak as the $(\text{M}+\text{H})^{++}$ for NCNO. What was confounding about these LC/MS results was the fact that NHNC, a suspected impurity in the NCNO sample, was readily observed but NCNO was not, even though norcocaine nitroxide had been demonstrated to be stable in neutral aqueous solutions at room temperature on the time scale of these experiments.

To ascertain whether a nitroxide and its corresponding hydroxylamine could be separated by LC under these conditions, the model nitroxide, TEMPO benzoate, and its reduction product, 1-hydroxy-TEMPO benzoate, were analyzed by LC/MS. Since the 1-hydroxy-TEMPO benzoate was not commercially available, it was prepared by a hydrogenation reaction of TEMPO benzoate over platinum oxide (courtesy of Ravi Oruganty of Dr. Dennis Wright's group). Using the same chromatographic conditions listed above, a 10 μL injection of 0.5 $\text{ng}/\mu\text{L}$ 1-hydroxy-TEMPO benzoate plus 5 $\text{ng}/\mu\text{L}$ TEMPO benzoate was analyzed by LC/ESI-MS. Figure 5-9 displays the ion chromatograms consistent with the $(\text{M}+\text{H})^{++}$ ion of TEMPO benzoate (m/z 277) and the $(\text{M}+\text{H})^+$ ion of 1-hydroxy-TEMPO benzoate (m/z 278). The peak at a retention time of 13.86 min. is the 1-hydroxy-TEMPO benzoate; however, there are two peaks in the ion chromatogram of m/z 277. LC/MS/MS of these eluting components revealed that the peak at 12.71 min. is consistent with TEMPO benzoate and confirmed that the peak at

13.86 min. is 1-hydroxy-TEMPO benzoate (data not shown). From these LC/MS and LC/MS/MS data, this model nitroxide and its corresponding hydroxylamine are easily separated under these reversed-phase LC conditions. Also, TEMPO benzoate does not appear to be reduced to a significant degree at the ESI emitter based on the relative abundance of the ^{13}C isotope (m/z 278) of the 12.71 min. peak $((5.2 \times 10^5 / 2.6 \times 10^6) 100\% = 20\%$; theoretical = 17.6%).

Revisiting the separation of norcocaine nitroxide and N-hydroxynorcocaine, an LC/MS experiment was conducted in which NCNO was injected onto column at a high organic content to test whether NCNO could pass through the C_{18} column intact. Figure 5-10 shows that when a 5 ng/ μL solution of NCNO was injected into a stream of 95% methanol and swept through the LC column, the $(\text{M}+\text{H})^{+\bullet}$ and $(\text{M}+\text{Na})^{+\bullet}$ ions of NCNO at m/z 305 and 327 were observed along with a large contribution from the $(\text{M}+\text{H})^+$ of NHNC at m/z 306 (Figure 5-10B). This proved that NCNO was stable enough to move through the LC column, yet it did not address whether NCNO and NHNC could be separated on a C_{18} column.

In an experiment designed to provide partial separation of these N-oxidative metabolites and keep them on the LC column for as brief a time as possible and minimize any potential degradation of norcocaine nitroxide, a 5 ng/ μL solution of NCNO was analyzed by LC/MS using a gradient of 60-100% B (95% methanol) in 2 min. Due to the large swept volume of the LC pump, NCNO (m/z 305 + 327) did not elute until 5.60 min. (Figure 5-11), but it is clearly separated from NHNC (m/z 306), which elutes at 8.10 min. However, there are two critical points to address from these data. First, the absolute intensity of the NHNC peak is greater than that of the NCNO peak, which was not the

case for the m/z 305 to m/z 306 ratio observed via direct infusion ESI-MS of NCNO in 50% methanol (compare to Figure 5-5A). Second, close inspection of the m/z 306 ion chromatogram in Figure 5-11 reveals that that component demonstrates a marked amount of peak fronting after the elution of NCNO at 5.60 min. The hypothesis drawn from these results was that norcocaine nitroxide was being converted to N-hydroxynorcocaine as it passed through the LC column. Specifically, NCNO appears to have been catalytically reduced to NHNC by interaction with free silanol sites on the silica particles of the stationary phase. The interaction between these free silanol groups, which contain moderately acidic protons (i.e., Si-OH), may be responsible for transforming NCNO to NHNC as norcocaine nitroxide partitions through the LC column. Hence, as NCNO is reduced to NHNC on the column, the product N-hydroxynorcocaine remains at the site of the reaction on the stationary phase, since NHNC has a greater capacity factor than NCNO under these chromatographic conditions (Figure 5-11). Eventually as the solvent strength increases, the NHNC produced *in situ* elutes from the column for detection by ESI-MS.

Since it appeared that NCNO was reacting as it passed through the LC column, it became necessary to change the experimental conditions in order to assay NCNO by LC/MS/MS. Three possible solutions were considered to solve this problem: chemical derivatization, changing the type of LC column, and addition of a suitable buffer to the mobile phase. The last of these options was easiest to implement and therefore the first to be attempted. As previously demonstrated, NCNO was proven to be stable in aqueous solution buffered with ammonium acetate (see Figure 5-5B). The expectation was that this buffer, when added to the mobile phase, could act as a pseudo ion-pairing agent by

preventing direct interaction between NCNO and any free silanol sites of the stationary phase.

Figure 5-12 displays the LC/MS chromatograms for the separation of 1 ng/ μ L NCNO with 0.2 ng/ μ L norcocaine and benzoynoregonine (the hydrolysis of the methyl ester of norcocaine) using 1 mM NH_4OAc in the mobile phase. Employing a linear gradient of 10-100% B in 9 min. (A=5% methanol + 1 mM NH_4OAc ; B=95% methanol + 1 mM NH_4OAc), all four metabolites of cocaine are easily distinguishable. A couple items to note from these data are that the peak width of NC is significantly greater than the other compounds, and that there is still evidence of the NCNO converting to NHNC on the column. However, the reduction of NCNO on column is greatly diminished when NH_4OAc is added to the mobile phase as compared to the LC/MS results when no modifier was used (compare to Figure 5-7 where the NCNO concentration is half that used in Figure 5-12), supporting the theory that the unmodified silanol groups are catalyzing this reaction.

To confirm that the peak fronting on the m/z 306 ion was indeed N-hydroxynorcocaine, an LC/MS/MS acquisition of a same sample used for Figure 5-12 was conducted. Time-segmented MS/MS for m/z 305 (8.6-9.6 min.) and m/z 306 (9.6-10.5 min.) yielded the data given in Figure 5-13. The MS/MS data for the peaks at 9.18 min. and 10.13 min. are displayed by the top and middle spectra, respectively, of Figure 5-13B. These product ion spectra confirm the structures of NCNO and NHNC, as illustrated by the fragmentation pathways for these metabolites in Figure 5-14. The bottom product ion spectrum of Figure 5-13B is an average of the MS/MS data for the m/z 306 precursor ion at the retention times between 9.61 and 9.79 min., which contains

the same principal fragment ions as in the middle product ion spectrum (i.e, m/z 152 and 184), indicating that the peak front to the 10.13 min. component is NHNC.

Effect of Mobile Phase Composition on Separation Efficiency

With a functional LC/MS/MS method developed, attempts were made to optimize the chromatographic separation of the N-oxidative metabolites of cocaine. Specifically, modifications to the mobile phase could help decrease the peak width and peak tailing of norcocaine, and may provide a greater chromatographic resolution between NC and NCNO (refer to Figure 5-12), even though the specificity of MS/MS does not require this. The two adjustments to the mobile phase that were explored were the concentration of the NH_4OAc buffer and the addition of acetonitrile (ACN) to the “strong” mobile phase (i.e., reservoir B). To accurately determine the effect of the mobile phase modifications, a constant gradient of 10-100% B in 9 min. was employed for all LC/MS experiments during which the mobile phase was altered. Tables 5-2 and 5-3 present an overview of the effects of changes to the mobile phase composition on the retention times (t_R), the peak widths (at baseline), and calculated resolutions for NCNO, NC and NHNC.

From the data in Table 5-2, increasing the concentration of ammonium acetate has a marginal improvement on the peak width of norcocaine. This may indicate that the dipole-dipole interactions between the secondary amine of NC (see Figure 5-1) and the acidic protons of the free silanol groups of the stationary phase are partially disrupted with mobile phases at higher ionic strengths. Nevertheless, the resolution between norcocaine and the other N-oxidative metabolites is compromised due mainly to the secondary interactions of NC with the stationary phase causing tremendous peak tailing as attributed by the large peak widths of NC (Table 5-2) and as was clearly illustrated in

Figure 5-12. At higher concentrations of NH_4OAc the NCNO/NC resolution also decreases to near zero due to the shift of the norcocaine retention time relative to norcocaine nitroxide. Interestingly, increases in the NH_4OAc concentration cause a noticeable decrease in the retention time of NC, whereas the retention times of NCNO and NHNC are essentially unaffected at the higher buffer concentrations. The resolution between N-hydroxynorcocaine and norcocaine nitroxide is, if anything, overresolved, with a resolution of greater than 1.5; however, note that the listed peak widths for NHNC neglect the peak fronting for this analyte.

The addition of acetonitrile to the strong mobile phase has a great effect on the chromatographic performance, as shown in Table 5-3. By fixing the amount of water at 5% (v/v) and concentration of NH_4OAc at 5 mM in the B mobile phase, increases to the volume percentage of ACN cause the elution times of NC and NHNC to decrease and concomitantly increase the retention time of NCNO. Additionally, the highest ACN concentration slightly improves the peak widths of all three N-oxidative metabolites of cocaine, and provides adequate chromatographic resolution ($R > 1.2$) between NCNO and NHNC. While the resolution between norcocaine and norcocaine nitroxide is still less than optimal when the B mobile phase contains 40% ACN, the retention time differential of 0.52 min. between these compounds reduces the cross contamination between NC and NCNO due to the tailing of the NC peak, as illustrated in Figure 5-15. For purposes of future quantitative LC/MS/MS analyses, the peak tailing of norcocaine will need to be addressed (see next section).

Based on the chromatographic results reported in Tables 5-2 and 5-3, all LC/MS(/MS) experiments described below use the following mobile phase compositions:

A=5% methanol with 2.5 mM ammonium acetate, B=55% methanol, 40% acetonitrile with 2.5 mM ammonium acetate. Close inspection of Table 5-2 shows that there is little difference in the chromatographic performance of NC, NCNO and NHNC at 2.5 versus 5.0 mM ammonium acetate. Therefore, the reduced concentration of NH_4OAc is employed to improve the ESI-MS signal stability without compromising the chromatography.

Evaluation of a Polymer-Based Column

In recent years, reversed-phase stationary phases based on polymer substrates instead of silica particles have gained wider acceptance in the field of liquid chromatography.^{179,233-235} One of the primary advantages of polymeric-based stationary phases is that they can be used across a wider range of pH ranges than silica-based stationary phases. For example, polymeric columns reportedly can function well using basic mobile phases (e.g., pH 8-12), whereas the ester linkages between the silanol backbone and the stationary phase moieties (e.g., C_{18}) are rapidly hydrolyzed from silica particles above pH 8. Another advantage of polymeric columns is the reduction or elimination of the secondary interactions between compounds with polar functionalities and the underivatized sites on the stationary phase often observed with silica substrates.

The previous LC/MS data have indicated that there is a significant problem of secondary interactions of the free silanol sites of the C_{18} column with some of the cocaine metabolites investigated. In the case of norcocaine, the interaction between unprotected silanol groups and the secondary amine of NC is likely causing the marked tailing of its chromatographic peak, as seen in Figures 5-12 and 5-15. Also, as hypothesized

previously, norcocaine nitroxide may be reduced to N-hydroxynorcocaine as it moves through the LC column via the interactions of the radical with the acidic silanol protons.

In an attempt to alleviate both of these experimental issues, a column packed with polyvinyl alcohol (PVA)-based particles modified with C_{18} stationary phase was obtained and tested. Since the polymer-based column has the same reversed-phase functionality as the silica-based LC column, few alterations to the developed LC/MS method were necessary.

Employing a shallower gradient of 7.5% per minute (i.e., 10-100% B in 12 min.) in order to increase the resolution between norcocaine and norcocaine nitroxide, Figure 5-16 displays the ion chromatograms for NC, NCNO and NHNC for LC/MS acquisitions using a silica-based (Figure 5-16A) and polymer-based (Figure 5-16B) column. What should be immediately evident from these data is that the tailing on the NC peak is markedly reduced on the polymeric-based column and that norcocaine's retention time decreases relative to NCNO. The peak width of norcocaine on the polymer column is 0.95 min. as compared to 1.76 min. on the silica C_{18} column. Reduction of the NC peak width coupled with the decreased retention time results in an improvement in the NC/NCNO resolution from 0.51 on the silica-based column to 1.87 on the polymeric C_{18} column. These findings support the conclusion that the secondary interactions between NC and the free silanol groups were causing the significant asymmetry in the norcocaine chromatographic peak.

Inspection of the LC/MS data for the polymer C_{18} column in Figure 5-16B indicates that there may still be reduction of NCNO to NHNC as it passes through the column; however, the extent of reduction is difficult to assess due the satellite ^{13}C

isotopic contribution of NCNO obscuring the peak fronting of the $(M+H)^+$ of NHNC (both at m/z 306). To better evaluate whether or not this reaction is still occurring on the polymeric-based column, an LC/MS acquisition of norcocaine nitroxide was conducted using isocratic conditions. Figure 5-17 displays the ion chromatograms for m/z 305, the $(M+H)^{++}$ of NCNO, and m/z 306, the $(M+H)^+$ of NHNC, when a 5 ng/ μ L solution of norcocaine nitroxide was injected onto the polymer column using a mobile phase of 65% B. As evident by the elevated baseline in the m/z 306 ion chromatogram between the retention time of NCNO (7.42 min.) and NHNC (9.60 min.), there appears to be a noticeable amount of N-hydroxynorcocaine produced from norcocaine nitroxide as it partitions through the polymer-based column just as NCNO does in a column packed with silica particles. This finding indicates two possibilities: either the unmodified vinyl alcohol protons are acidic enough to catalyze the reduction of NCNO to NHNC, or the norcocaine nitroxide is reacting with another component in the LC column.

Even though the conversion of NCNO to NHNC was not eliminated on the polymer-based column as anticipated, the reduction of the peak tailing on norcocaine on this column is advantageous. Therefore, in the final assessment, the polymeric C_{18} column will be employed for the remainder of the LC/MS and LC/MS/MS experiments reported here. Furthermore, the LC gradient chosen for the extraction and *in vitro* assays is the 7.5% per minute linear ramp (i.e., 10-100% B in 12 min.) as it provides baseline resolution ($R>1.5$) for the three targeted N-oxidative metabolites of cocaine.

Extraction of N-Oxidative Metabolites of Cocaine

In order to collect the cocaine metabolites from the microsomal protein suspension and eliminate potential interferences during LC/MS/MS analyses, an

extraction method is necessary. The two methods tested for capturing the targeted analytes NC, NCNO and NHNC were liquid/liquid extraction (LLX) and solid phase extraction (SPE).

The liquid/liquid extraction method employed was developed by Roberts and coworkers for the extraction of cocaine, cocaethylene, norcocaine and benzoylecgonine.²³⁶ Based on their results, the extraction efficiency for NC was 72.4% and between 75 and 85% for cocaine and its other analogs. To ensure the LLX method was viable for norcocaine nitroxide and N-hydroxynorcocaine prior to *in vitro* incubations, the three targeted N-oxidative metabolites at a concentration of 100 μ M (100 nmol/mL) were added to 1 mL of 50 mM TRIS buffer solution containing 2 mM NADPH (nicotinamide adenine dinucleotide phosphate [reduced]), the cytochrome P450 enzyme cofactor, and 10 mM NaF, an esterase inhibitor. Test solutions were prepared with the individual metabolites and with a combination of NC, NCNO and NHNC for a total of four samples. To the 1 mL TRIS buffer solution a 10 mL aliquot of 9:1 (v/v) methylene chloride/isopropanol was added and vortexed for 60 s. After standing for 5 min., the sample vials were again briefly vortexed then centrifuged for 5 min. at 1000 g. Eight milliliters of the organic layer (bottom layer) were pipetted off and split into two 4 mL fractions. The purpose for the second fraction of each sample was to use that fraction for solid phase extraction (see below). The organic solvent was evaporated under a stream of nitrogen and the LLX samples were stored at -20° C until analysis by LC/MS.

Prior to assay by LC/MS, the LLX samples were reconstituted in 20% methanol with 5 mM NH_4OAc , vortexed, filtered through a 0.45 μ m Teflon filter and diluted 1 to 10 in the A mobile phase (5% methanol + 2.5 mM NH_4OAc). The purpose for diluting

the samples was to ensure that the concentrations of the N-oxidative metabolites were in a reasonable range such that a good response could be obtained by LC/MS and yet not overload the LC column. To elaborate, if the extraction method described above was 100% efficient, the concentrations of the reconstituted LLX solutions would have been 11.6-12.2 ng/ μ L, which was approximately ten times greater than the concentrations of the standards used during the LC/MS method development.

The LC/MS results of the combination liquid/liquid extraction sample are displayed in Figure 5-18. The peaks for NC at 11.24 min. and NHNC at 13.13 min. are clearly observed; however, NCNO (m/z 305 + 327) cannot be distinguished from the background at its predicted retention time (12.2-12.4 min.). Examination of the LLX sample containing only NCNO yielded similar results (Figure 5-19). Norcocaine and N-hydroxynorcocaine are observed in the NCNO extraction sample since they are known contaminants in the NCNO standard. Nevertheless, it was disconcerting to be able to easily observe NC and NHNC from the LLX experiments and yet not detect NCNO.

It is a reasonable assumption that these three metabolites should have similar extraction efficiencies since they are much more soluble in organic solvents than water. Even though the ionization efficiency of NCNO is about ten times less than that of NHNC (data not shown), the LC/MS response of NHNC in Figure 5-18 suggests NCNO should have been observed above the noise level unless either the extraction efficiency of NCNO is much less than NC and NHNC, or norcocaine nitroxide is lost by chemical means (e.g., reaction with a compound) or physiochemical means (e.g., adsorption to a surface).

Nitroxides are known to be good oxidizing agents.^{230,232} Hence, it was suspected that NCNO might be reduced to NHNC in the presence of NADPH, the cytochrome P 450 cofactor responsible for oxidizing foreign compounds in living systems (e.g., in liver microsomes). To test this hypothesis, a solution of 5 ng/ μ L NCNO containing 15 ng/ μ L NADPH was examined by direct infusion ESI-MS, where the ion intensities for NCNO and NADPH were measured as a function of time. Figure 5-20 shows the ion chromatograms for NCNO (A), NHNC (B), NADPH (C) and NADP (D) over the course of 30 min. Based on these data, there is no significant change in the ratios for NCNO/NHNC or for NADPH/NADP, indicating that the redox reaction between NCNO and NADPH does not occur rapidly, if at all, at room temperature in the absence of cytochrome P450.²³⁷

Two other hypotheses as to why NCNO was lost during the initial LLX experiments are that norcocaine nitroxide may be reacting with the TRIS buffer, which had been previously demonstrated to consume hydroxyl radicals,²³⁸ or that NCNO might be binding to the Teflon filter, which works best with organic solutions. To test both theories simultaneously, a second set of LLX experiments was conducted in which NCNO was added to 0.5 mL solutions of 5 mM NH_4OAc , 50 mM TRIS and 25 mM $\text{K}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ to a final concentration of 50 $\mu\text{g/mL}$. After reconstitution in 2 mL 20% methanol with 5 mM NH_4OAc , the LLX of NCNO from ammonium acetate was filtered through a 0.45 μm Teflon filter while the extracts from TRIS and the phosphate buffer were filtered through a 0.45 μm nylon filter.

Ten microliters of the filtrate solutions were injected and analyzed by LC/MS. The results of the LLX experiments from NH_4OAc , TRIS and phosphate buffered

solutions are given in Figures 5-21, 5-22, and 5-23, respectively. Note that the longer retention times of NC, NCNO and NHNC for the LLX sample from NH_4OAc (Figure 5-21) are due to a decrease in the LC column flow rate that was corrected before LC/MS of the other LLX samples (Figures 5-22 and 5-23). Upon inspection of these LC/MS data, two conclusions are evident. First, NCNO can be efficiently extracted from TRIS buffered solutions, as well as from aqueous solutions buffered with phosphate. Second, passing NCNO through a Teflon filter markedly reduces the recovery of norcocaine nitroxide. This point is better illustrated by comparing the area ratios of NCNO/NC and NCNO/NHNC relative to the area ratio of NHNC/NC, as listed in Table 5-4. It has already been demonstrated that NCNO is stable in NH_4OAc solutions (see Figure 5-5), and yet its recovery relative to that of the NC and NHNC impurities in the sample is 4-6 times lower than that observed for the LLX of NCNO in TRIS and phosphate buffered solutions. Since the type of buffer added to the aqueous solution should have little to do with the partitioning of an analyte into another phase if the pHs are similar (TRIS and phosphate are pH 7.4, NH_4OAc is approximately pH 7), then the logical conclusion is that norcocaine nitroxide is adsorbing to the Teflon filter. This also explains why NCNO was not observed in the preliminary LLX experiments, as all those samples were filtered through a Teflon filter.

Solid phase extraction was also conducted on the original LLX samples, which were split into two fractions (see above). The advantage of SPE is that the targeted analytes can be more selectively recovered than by LLX alone, providing a cleaner sample for LC/MS analyses. Briefly, 1 mL SPE tubes (3M) packed with C_{18} stationary phase were conditioned with 300 μL 95% methanol plus 5 mM NH_4OAc followed by

300 μL 5 mM NH_4OAc . The LLX fractions, reconstituted in 0.5 mL 20% methanol with 5 mM NH_4OAc were loaded into the SPE tubes and allowed to partition into the stationary phase under a slight vacuum (20 in. Hg). The sample SPE tubes were washed with 300 μL volumes of 5 mM NH_4OAc and 20% methanol with 5 mM NH_4OAc . Elution of the analytes was achieved by addition of 200 μL 95% methanol plus 5 mM NH_4OAc with the assistance of a moderate vacuum (15 in. Hg) and collected in 0.6 mL polypropylene tubes. The recovered eluent was filtered with a 0.45 μm Teflon filter and diluted 1:5 with 5% methanol plus 2.5 mM NH_4OAc (mobile phase A) for LC/MS assay.

Unfortunately, the marked loss of NCNO via filtration with a Teflon filter was not discovered prior to the preparation of the SPE samples. This is reflected in the LC/MS results of Figure 5-24, where the mixture of NCNO, NC and NHNC underwent the SPE procedure described above. Again, the peaks for norcocaine and N-hydroxynorcocaine are clearly evident at the retention times of 11.20 and 13.15 min., respectively, yet norcocaine nitroxide cannot be distinguished from the background with reasonable confidence (the peak at 12.30 min. is consistent with the retention time of NCNO). Although NCNO was not readily observed following SPE, it was believed that this was a consequence of the Teflon filter and not due to a low recovery from the SPE tube; however, this issue will be addressed again below.

In Vitro Incubations of Norcocaine and N-hydroxynorcocaine

It has been suggested that the hepatotoxicity of cocaine is due to the production of norcocaine nitroxide via oxidative metabolism (see Figure 5-1).^{208,213} To date, the presence of NCNO by *in vivo* cocaine metabolism has not been demonstrated and has only been observed by EPR spectroscopy of *in vitro* incubations of NHNC in rat liver

microsomes.²⁰³⁻²⁰⁵ Hence, this section focuses on the application of the LC/MS(/MS) and extraction methods described above in an attempt to confirm whether NCNO is indeed produced *in vitro*.

Rat liver microsomes were prepared by John Munson at the University of Florida Environmental and Human Toxicology Lab and stored at -80°C prior to use. Into 150×16 mm glass tubes an aliquot of liver microsomes containing 4 mg cytochrome P450, as determined by the Lowry assay, along with 2 mM NADPH, 10 mM NaF and $100\text{ }\mu\text{M}$ of either norcocaine or N-hydroxynorcocaine were added to a total volume of 2 mL in 25 mM phosphate buffer (pH 7.4). Sodium fluoride was added as an inhibitor to the esterases in the liver microsomes to maximize the oxidative metabolism of NC and NHNC. The *in vitro* suspensions were incubated at 37°C in a shaking water bath for a period of 15 min. The reaction was terminated by addition of 5 mL of 9:1 methylene chloride/isopropanol and placing the glass vials into a container of ice. After 1 min., the organic solvent/protein suspension was vortexed for 30 seconds then replaced in the ice bath for another 5 min. The incubation mixture was again briefly vortexed and centrifuged for 10 min. at $1,500\text{ g}$ to ensure phase separation. A total of 4 mL of the organic (bottom) layer was removed and split into two 2 mL fractions. The purpose for the second fraction was for the possibility of executing SPE on this set of samples. Evaporation of the organic solvent was conducted under a stream of nitrogen while the sample vials were immersed in a water bath at 35°C . Samples to be used later (e.g., for SPE) were stored at -20°C . The "fraction 1" samples (i.e., the samples treated solely by LLX) were reconstituted in $100\text{ }\mu\text{L}$ 20% methanol with 5 mM NH_4OAc , vortexed, filtered with a $0.45\text{ }\mu\text{m}$ nylon filter and stored at -20°C until examined by LC/MS(/MS).

Figure 5-25 displays the results of the LC/MS analysis of the 100 μ M norcocaine *in vitro* incubation following a 1:10 dilution of the reconstituted LLX sample. There are four items to note from these data. First, NCNO cannot be observed in this sample, partly due to the fact that a two-step oxidation is required to convert NC to NCNO (Figure 5-1). Second, the primary oxidation product, N-hydroxynorcocaine, is observed at 10.79 min. as well as two other earlier eluting products (t_R = 5.92 and 6.36 min.) at the same m/z (306), which was later confirmed to be (m,p)-hydroxynorcocaine by MS/MS (see below). Third, the peak eluting at 4.85 min. corresponds to benzoynoregonine (BNE), the hydrolysis of the methyl ester of NC. Benzoylcegonine, the metabolite arising from hydrolysis of cocaine's methyl ester, is known to be produced by liver esterases,¹⁹⁷ and therefore it is logical to assume that NC would also be biotransformed to BNE via esterases in the liver microsomes. Addition of NaF should have inhibited these esterases during these experiments; however, the microsomal esterases might not have been completely inactivated. The observation of BNE during this *in vitro* experiment may suggest that benzoynoregonine is also produced by a cytochrome P450 isozyme. The fourth interesting item from these LC/MS data is the observation of a peak at m/z 304 at the retention time of 7.78 min. Based on the LC/MS/MS data (see below) and the retention time of a standard (data not shown), this biotransformation product is cocaine. The observation of cocaine from an *in vitro* incubation of NC is perplexing as it requires the reverse reaction from that executed by cytochrome P450. Perhaps there is an N-methyltransferase in the liver microsomes responsible for producing cocaine from norcocaine.

For reasons that will be discussed below, the second fraction of the 100 μM NC *in vitro* incubation was subjected to solid phase extraction, and the LC/MS analysis of this sample was repeated to confirm the presence of the metabolites observed from Figure 5-25 (data not shown). An overview of the LC/MS/MS results from the analysis of the selected ions displayed in Figure 5-25 is given in Table 5-5. As discussed in the previous paragraph, the MS/MS data from the eluting components with ions at m/z 276, 304 and 306 ($t_R = 10.79$ min.) all yielded a neutral loss of 122 u , the benzoic acid moiety, confirming that the structures of these ions are consistent with benzoylecgonine, cocaine and N-hydroxynorcocaine, respectively. The product ion spectra for the two closely eluting peaks at retention times about 6.0 min. with a precursor m/z 306 were identical, giving a major fragment at m/z 168 and a minor fragment ion at m/z 136, which is consistent with the loss of hydroxybenzoic acid and a sequential loss of methanol. Based on these data, these metabolites are m- and p-hydroxynorcocaine; however, without pure standards of each, the isomeric form cannot be assigned. Finally, the earlier eluting ion at m/z 290 ($t_R = 6.56$ min.) does not seem to be consistent with a biotransformation product of NC ($t_R = 8.86$ min., also m/z 290) as its MS/MS data reveal only the loss of water (m/z 272) and methanol (m/z 258).

Since the toxic metabolite norcocaine nitroxide could not be observed from norcocaine *in vitro*, 100 μM N-hydroxynorcocaine was also incubated in rat liver microsomes. The LC/MS assay of that sample is given in Figure 5-26. Inspection of the ion chromatogram for the $(M+H)^{+*}$ and $(M+Na)^{+*}$ of NCNO (m/z 305 and 327) does not reveal the presence of NCNO. In fact, only the hydrolysis metabolites of benzoylecgonine and norcocaine are observed at the retention times of 4.87 and 8.91

min., respectively. These results suggest that the liver microsome esterases are still active even in the presence of 10 mM NaF. Furthermore, if NCNO is indeed being produced by *in vitro* incubation of NHNC, then possible reasons that NCNO is not being observed are that its biotransformation efficiency is low, it is binding irreversibly to the microsomal proteins, or NCNO is lost during sample recovery and sample handling procedures.

To attempt to overcome these limitations and to gain better insight into the processes occurring during the *in vitro* incubations, a new set of experiments was conducted in which NHNC was incubated at a concentration five-times higher than previously done (500 μ M), and NCNO was incubated with and without liver microsomes at 100 μ M. Following the extraction of the metabolites with 9:1 methylene chloride/isopropanol, SPE was performed. This was deemed necessary since in the previous set of microsomal samples, where only LLX was employed, a significant amount of high mass (625-850 u), non-polar components were eluting from the column for a period of 15-60 min. following LC/MS analyses. It is suspected that these ubiquitous compounds were phospholipids, which, over time, would have concentrated on the LC column, affecting the chromatography and shortening the column's lifetime. Finally, during these LC/MS(/MS) experiments, the injection volume was increased from 10 to 20 μ L to improve detection limits.

During the SPE experiments conducted on the norcocaine nitroxide standard described above, NCNO was not observed by LC/MS; it was theorized that this was due to NCNO adsorbing to the Teflon filters used during those experiments. Therefore, to test that hypothesis, 100 μ M NCNO was added to 1 mL of the phosphate buffer,

extracted with methylene chloride/isopropanol, the organic solvent evaporated, and the recovered solid was reconstituted in 0.5 mL 20% methanol with 5 mM NH_4OAc for SPE using the same procedure as described above. The methanolic eluant solution from the SPE procedure was evaporated, and the remaining solid residue was dissolved in 100 μL 20% methanol plus 5 mM NH_4OAc . Before LC/MS analysis, the reconstituted SPE sample was filtered via centrifugation at 2,500 g for 3 min. through a 0.45 μm nylon filter membrane contained in a centrifuge tube.

The LC/MS results of the SPE of NCNO are given in Figure 5-27. What is evident from these data is that the extraction efficiency for NCNO is poor compared to the norcocaine and N-hydroxynorcocaine impurities in the sample. LC/MS analysis of the NCNO standard prior to SPE indicated that the absolute intensities of NC and NCNO were nearly equivalent (data not shown), yet for the SPE of NCNO, the intensity of the NCNO peak (10.32 min.) is less than 10% that of the NC peak (Figure 5-27). The reason for such a low recovery of NCNO was not initially clear, since NCNO seemingly undergoes LLX with reasonable efficiency (see Figures 5-22 and 5-23), and NCNO chromatographs predictably on a C_{18} column. Upon review of the manufacture's specifications of the SPE cartridges it was discovered that the membrane supports containing the stationary phase is composed of 10% Teflon. As previously mentioned, it is believed that NCNO binds strongly to Teflon, causing a severe bias against the recovery of norcocaine nitroxide.

Even though the recovery of NCNO is low following SPE, the other *in vitro* samples had already been subjected to SPE and were therefore analyzed by LC/MS(/MS). Not surprising, the LC/MS data in Figure 5-28 from the incubation of 100 μM NCNO in

liver microsomes shows only a trace of recovered NCNO (t_R = 10.53 min.).

Unfortunately, it cannot be concluded at this time whether this result is due mainly to the irreversible binding of NCNO to microsomal proteins or to the loss of NCNO during SPE.

The LC/MS results from the 500 μ M *in vitro* incubation of NHNC are displayed in Figure 5-29. What was encouraging was the observation of a small peak at 10.56 min. for the m/z 305 + 327 ion chromatogram (Figure 5-29E), which may signify the presence of NCNO. Another tentative oxidative metabolite of N-hydroxynorcocaine observed in these data was the partially resolved peaks at 8.94 and 9.28 min. in the m/z 322 ion chromatogram (Figure 5-29C). These peaks could represent *m*- and *p*-hydroxy-N-hydroxynorcocaine. Some other interesting findings from these LC/MS data are observation of peaks corresponding to hydrolysis metabolites benzoynorecgonine (Figure 5-29A) and norcocaine (Figure 5-29D), and a peak at 8.39 min. represented by m/z 304 (Figure 5-29B), which was demonstrated in the *in vitro* incubation of NC to be cocaine.

To verify the presence of NCNO and confirm the assignments of the other metabolites observed in Figure 5-29, LC/MS/MS was performed on the NHNC *in vitro* sample. The time-segmented precursor-to-product ion chromatograms for the targeted biotransformation products are displayed in Figures 5-30A-G with their corresponding product ion mass spectra shown in Figures 5-31A-G. As indicated by the small peak at 10.63 min. in Figure 5-30F, norcocaine nitroxide is indeed a product formed by oxidative metabolism of N-hydroxynorcocaine. The representative MS/MS data for this peak are given in Figure 5-31F. While the product ion spectrum is noisy, the observation of the

ions at m/z 273 and m/z 183, the loss of methanol and benzoic acid respectively, do correlate to NCNO as previously demonstrated by the LC/MS/MS data for NCNO in Figure 5-13B.

Based on the other product ion spectra in Figure 5-31, the assignment for the other metabolites are given in Table 5-6. Referring back to the LC/MS data in Figure 5-29C, the partially resolved peaks at m/z 322 do yield two different product ion spectra by LC/MS/MS (Figures 5-31D and E). The later eluting component, metabolite E, has a product ion spectrum (Figure 5-31E) consistent with (m,p)-hydroxy-N-hydroxynorcocaine as a result of the loss of hydroxybenzoic acid (138 u) from the precursor $(M+H)^+$ ion at m/z 322. However, a structure based on the metabolism of NHNC cannot be readily predicted for "metabolite D", which primarily yields a loss of 120 u from the precursor m/z 322 ion (Figure 5-31D). Therefore, the identity of this compound and whether it is a biotransformation product of NHNC is currently unresolved.

In summary, the LC/MS/MS method developed here is suitable for the analysis of the N-oxidative metabolites of cocaine, as well as many of its hydrolysis metabolites (e.g., benzoynorecgonine). *In vitro* incubation of norcocaine confirmed that N-hydroxynorcocaine is a major biotransformation product; however, norcocaine nitroxide could only be confirmed by incubation of N-hydroxynorcocaine in rat liver microsomes at a relatively high concentration ($500\text{ }\mu\text{M}$). The observation of NCNO from the *in vitro* incubation of NHNC is low likely due to a combination of low biotransformation efficiency, irreversible binding to microsomal proteins, and sample handling losses (e.g., binding to the Teflon membrane of SPE cartridge). Nevertheless, confirmation of the

presence of the hepatotoxic metabolite norcocaine nitroxide *in vitro* via LC/MS/MS lays the groundwork for many other future experiments to further characterize this rather elusive compound.

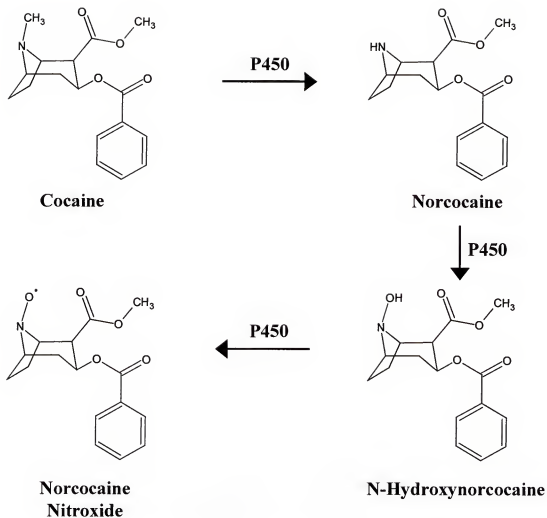


Figure 5-1: N-oxidative metabolic pathway of cocaine. Norcocaine nitroxide, a stable organic radical, has been demonstrated to be toxic to the liver.

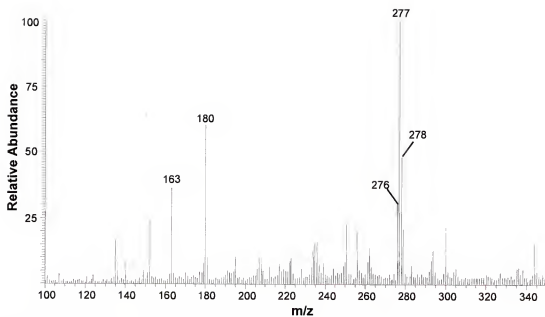


Figure 5-2: ESI-MS data for 2 ng/ μ L solution of TEMPO benzoate (MW = 276). This organic radical forms a significant M^+ ion (m/z 276) by positive ion ESI as a result of one-electron oxidation.

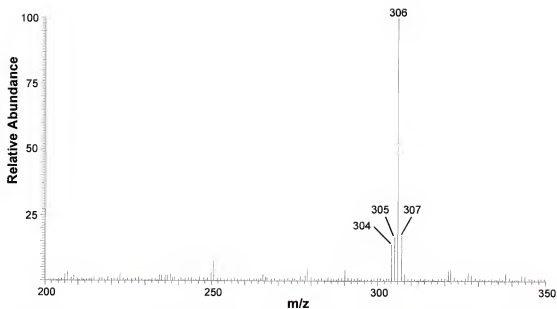


Figure 5-3: ESI-MS of norcocaine nitroxide (MW 304) in 50% methanol + 0.5% acetic acid. The M^+ and $(M+H)^{+•}$ ions of NCNO are minor relative to the ion at m/z 306, suspected to be the $(M+H)^+$ of the reduction product, N-hydroxynorcocaine.

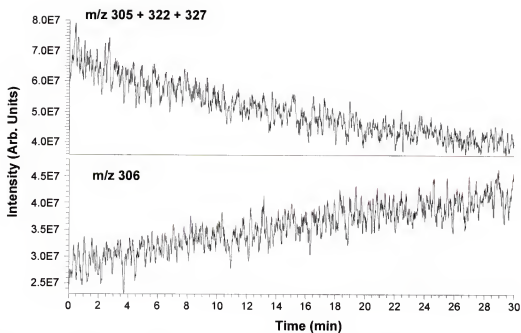


Figure 5-4: ESI-MS response for norcocaine nitroxide in 50% methanol + 0.1% acetic acid. The top chromatogram represents the $(M+H)^+$, $(M+NH_4)^+$, and $(M+Na)^+$ ions of NCNO. The bottom ion chromatogram is diagnostic of the $(M+H)^+$ of N-hydroxynorcocaine.

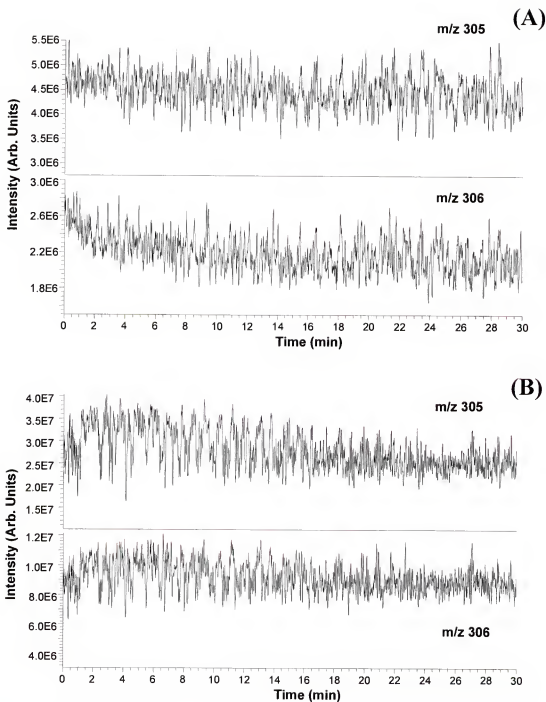


Figure 5-5: ESI-MS response of norcocaine nitroxide in aqueous-based solution near pH 7. (A) Ion chromatograms for $(\text{M}+\text{H})^{+\bullet}$ of NCNO (m/z 305) and $(\text{M}+\text{H})^+$ of NHNC (m/z 306) in 50% methanol. (B) Ion chromatograms for $(\text{M}+\text{H})^{+\bullet}$ of NCNO and $(\text{M}+\text{H})^+$ of NHNC in 50% methanol + 1 mM NH_4OAc .

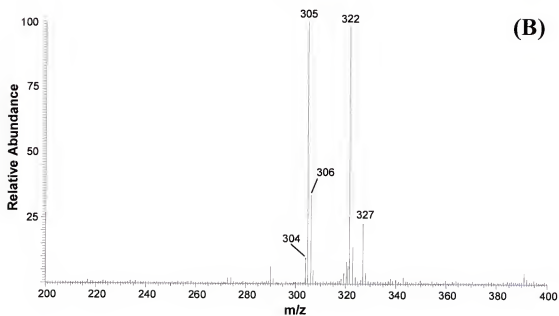
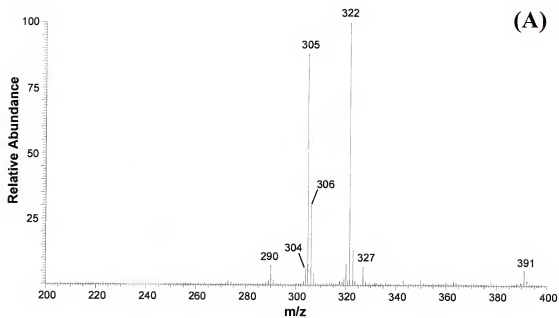


Figure 5-6: ESI-MS data of norcocaine nitroxide in 1 mM NH_4OAc . (A) Mass spectrum of NCNO after 0.5 h at room temperature. (B) Mass spectrum of NCNO after 18 h at room temperature.

Table 5-1: Normal operating parameters for the LCQ ion trap mass spectrometer during high flow rate LC/MS experiments.

LCQ Instrument Parameter	Typical Operating Voltage
ESI Needle Voltage	+4.0 kV
Sheath Gas Flow	55 (Arb. Units)
Auxiliary Gas Flow	30 (Arb. Units)
Heated Capillary Temperature	225° C
Heated Capillary Voltage	+10 V
ESI Tube Lens Offset	-40 V
Skimmer	Ground
Octopole 1 Offset	-4.5 V
Octopole 2 Offset	-7.0 V
Octopoles' AC amplitude	700 V _{p-p}
Interoctopole Lens Voltage	-16.0 V
Ion Trap Offset	-10.0 V

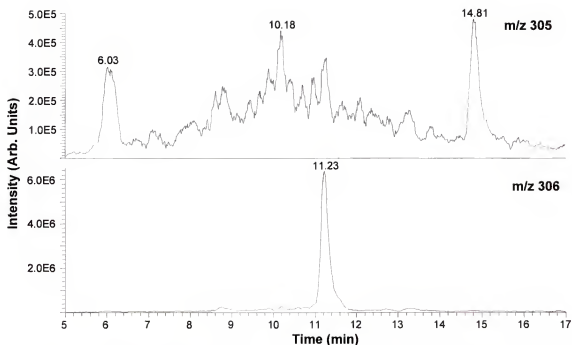


Figure 5-7: LC/MS ion chromatograms for 0.5 ng/ μ L norcocaine nitroxide using no mobile phase modifier. The peak at 11.23 min. in the m/z 306 ion chromatogram is consistent with N-hydroxynorcocaine. The peak at 10.18 min. (m/z 305) may be attributed to the $(M+H)^{+}$ of NCNO (see text for details).

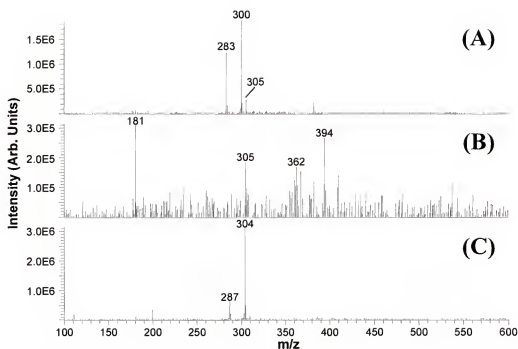


Figure 5-8: ESI-MS data of the major m/z 305 peaks from the LC/MS results in Figure 5-7. (A) Mass spectrum of peak at 6.03 min. displaying the m/z 305 ion as the $(M+Na)^+$ of an oligomer of polyethylene glycol. (B) Mass spectrum of m/z 305 component eluting at 10.18 min. The poor signal-to-noise of the m/z 305 ion in these data makes the designation of this eluting compound as norcocaine nitroxide problematic. (C) Mass spectrum of peak eluting at 14.81 min. Notice that the m/z 305 ion is a ^{13}C isotope of the base peak at m/z 304.

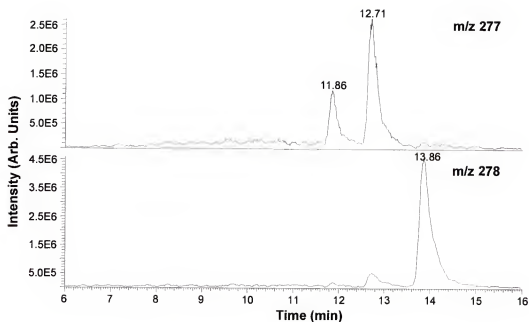


Figure 5-9: LC/MS results for TEMPO benzoate and 1-hydroxy-TEMPO benzoate mixture using water/methanol gradient with no mobile phase modifier. The peaks eluting at 12.71 min. and at 13.86 min. are TEMPO benzoate and 1-hydroxy-TEMPO benzoate, respectively. LC/MS/MS of this sample confirmed the assignments of these compounds and that the m/z 277 ion component eluting at 11.86 min. was an artifact.

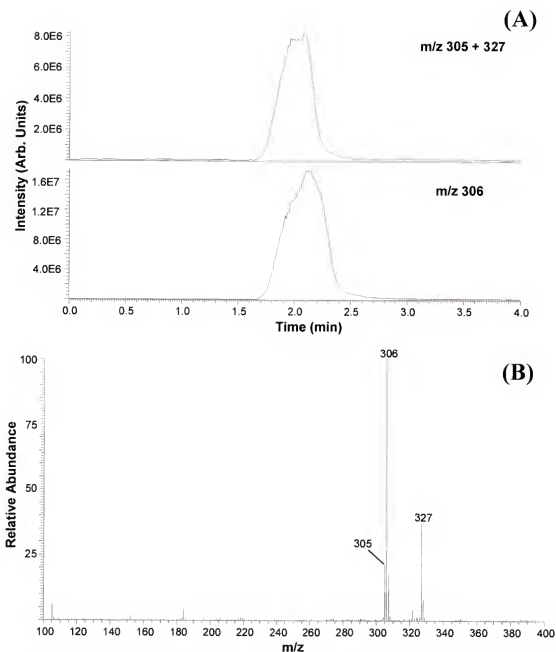


Figure 5-10: Isocratic LC/MS data for 5 ng/ μ L norcocaine nitroxide at 95% methanol. (A) The ion chromatograms for the $(M+H)^{++}$ and $(M+Na)^{+}$ ions of NCNO (top) and the $(M+H)^{+}$ ion of NHNC (bottom). (B) ESI mass spectrum for the 2.05 min. peak displaying norcocaine nitroxide representative ions at m/z 305 and m/z 327 and the $(M+H)^{+}$ of N-hydroxynorcocaine at m/z 306.

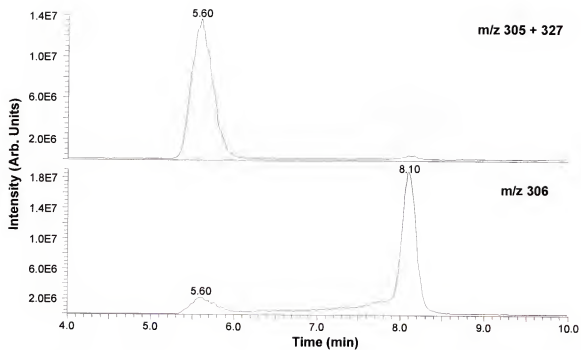


Figure 5-11: LC/MS of 5 ng/ μ L norcocaine nitroxide employing gradient of 60-100% B (95% methanol) in 2 min. Notice the large amount of peak fronting on the m/z 306 peak, which is believed to be caused by reduction of NCNO to NHNC as norcocaine nitroxide passes through the column.

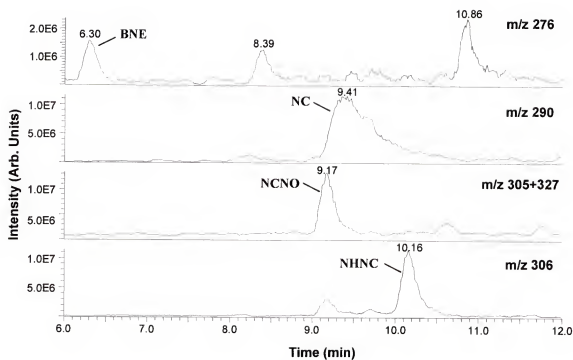


Figure 5-12: Gradient LC/MS of cocaine metabolite mixture employing 1 mM NH_4OAc in the mobile phase. The peaks designated benzoynoregonine (BNE), norcocaine (NC), norcocaine nitroxide (NCNO), and N-hydroxynorcocaine (NHNC) were verified by LC/MS/MS.

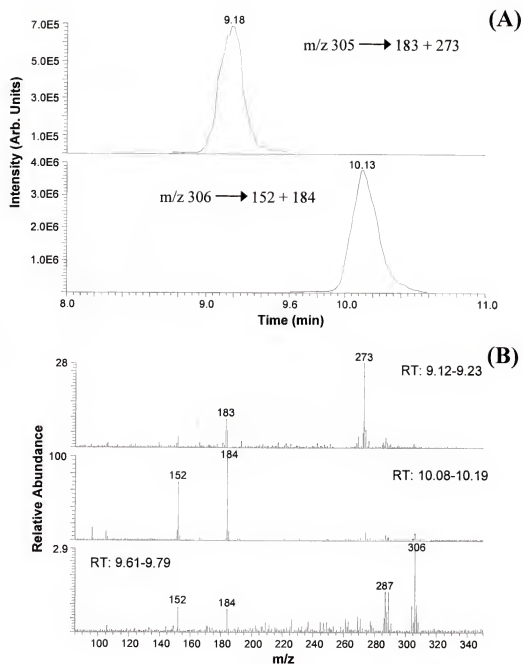
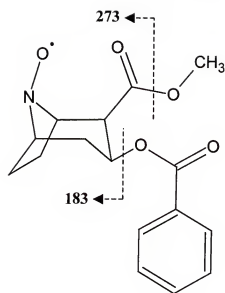
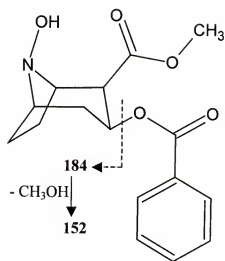


Figure 5-13: LC/MS/MS of sample analyzed in Figure 5-12. (A) Extracted ion chromatograms for described precursor-to-product ion transitions. (B) Product ion spectra for the noted retention times. See text for details.



Norcocaine nitroxide

$(\text{M}+\text{H})^{++}$ m/z 305



N-hydroxynorcocaine

$(\text{M}+\text{H})^+$ m/z 306

Figure 5-14: Fragmentation scheme for NCNO and NHNC by MS/MS.

Table 5-2: Effect of ammonium acetate concentration on the chromatographic response of the N-oxidative metabolites of cocaine. Note that the listed peak widths of NHNC neglect the peak fronting of that compound.

	1 mM NH ₄ OAc	2.5 mM NH ₄ OAc	5 mM NH ₄ OAc
t _{R, NCNO} (min.)	9.17	9.20	9.24
t _{R, NC} (min.)	9.39	9.17	9.18
t _{R, NHNC} (min.)	10.16	10.18	10.23
W _{NCNO} (min.)	0.52	0.46	0.46
W _{NC} (min.)	1.54	1.50	1.45
W _{NHNC} (min.)	0.64	0.59	0.63
R _{NC, NCNO}	0.21	0.03	0.06
R _{NC, NHNC}	0.71	0.97	1.01
R _{NCNO, NHNC}	1.71	1.87	1.82

Table 5-3: Effect of acetonitrile concentration in the strong mobile phase reservoir on the chromatographic response of the N-oxidative metabolites of cocaine. Note that the peak widths listed for NHNC neglect the peak fronting on this compound.

	10% ACN	20% ACN	40% ACN
$t_{R, NCNO}$ (min.)	9.19	9.23	9.31
$t_{R, NC}$ (min.)	8.96	8.89	8.79
$t_{R, NHNC}$ (min.)	10.09	10.03	9.93
W_{NCNO} (min.)	0.48	0.49	0.44
W_{NC} (min.)	1.50	1.45	1.34
W_{NHNC} (min.)	0.54	0.58	0.52
$R_{NC, NCNO}$	0.23	0.35	0.58
$R_{NC, NHNC}$	1.11	1.12	1.23
$R_{NCNO, NHNC}$	1.76	1.50	1.29

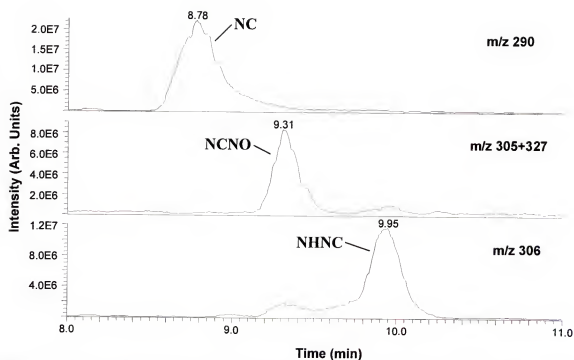


Figure 5-15: LC/MS of NC, NCNO and NHNC using 55% methanol, 40% acetonitrile plus 5 mM NH_4OAc as “B” mobile phase. A linear gradient of 10-100% B in 9 min. was employed.

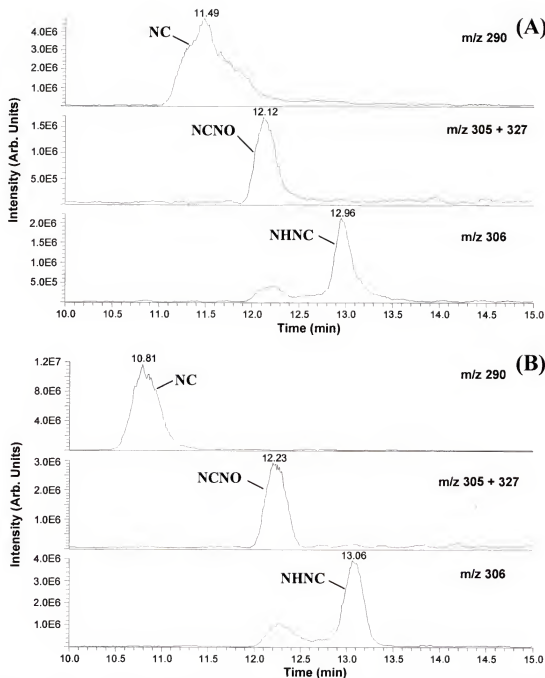


Figure 5-16: LC/MS of NC, NCNO and NHNC on columns of differing substrates. (A) Separation using silica-based C_{18} stationary phase. (B) Separation using a polymer-based C_{18} stationary phase. Notice the significant reduction in the retention time and in the amount of tailing on the norcocaine peak. This indicates that the modified silica particles are not fully endcapped, causing the secondary interactions between NC and free silanol sites.

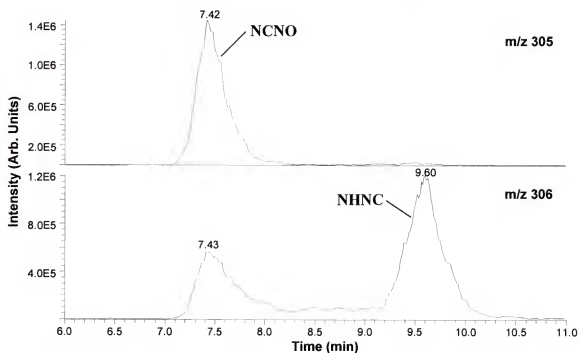


Figure 5-17: Isocratic LC/MS of 5 ng/ μ L NCNO on polymeric-based C_{18} column. The elevated signal at m/z 306 between the elution of NCNO and NHNC indicates that norcocaine nitroxide is being reduced to N-hydroxynorcocaine as it partitions through the polymer-based column.

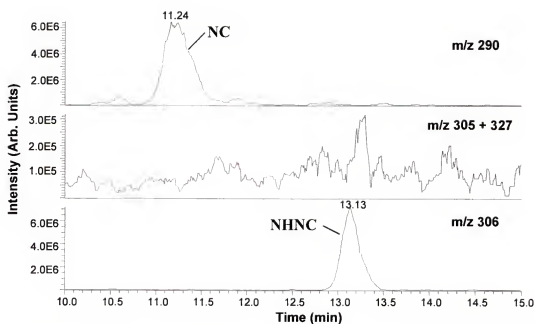


Figure 5-18: LC/MS results following liquid/liquid extraction of NC, NCNO and NHNC. The expected elution time for norcocaine nitroxide is 12.2-12.4 min.

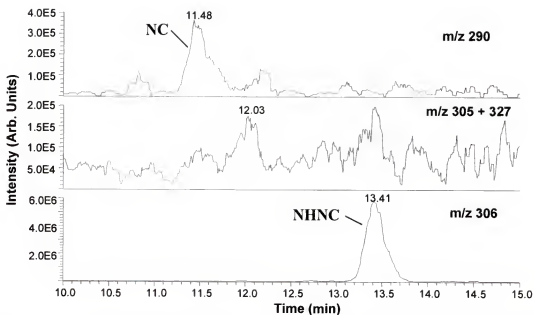


Figure 5-19: LC/MS results following LLX of norcocaine nitroxide. The expected elution time of NCNO is 12.5-12.7 min. Norcocaine and N-hydroxynorcocaine are observed since they are contaminants in the NCNO standard.

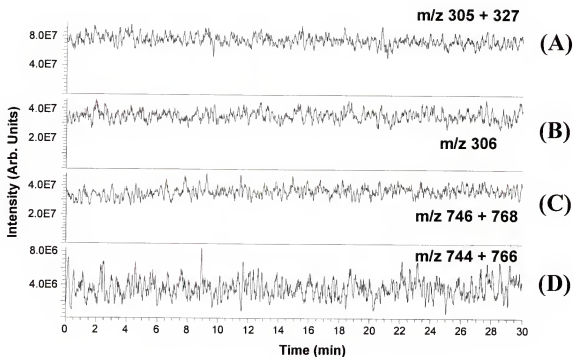


Figure 5-20: ESI-MS monitoring of 5 ng/ μ L norcocaine nitroxide plus 15 ng/ μ L NADPH. The ion chromatograms for NCNO (A), NHNC (B), NADPH (C) and NADP (D) show no change in absolute intensity within experimental error over 30 min. This supports the conclusion that NCNO is not reduced in the presence of NADPH.

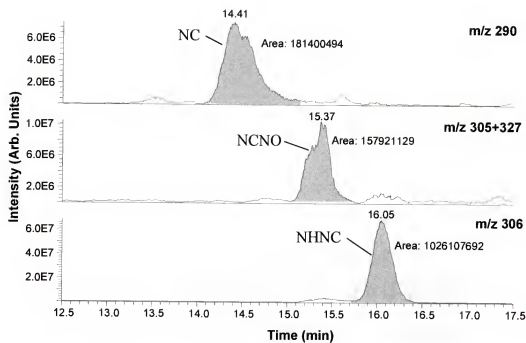


Figure 5-21: LC/MS results following LLX of norcocaine nitroxide from 5 mM NH_4OAc . Prior to analysis, the reconstituted solution was filtered through a 0.45 μm Teflon filter.

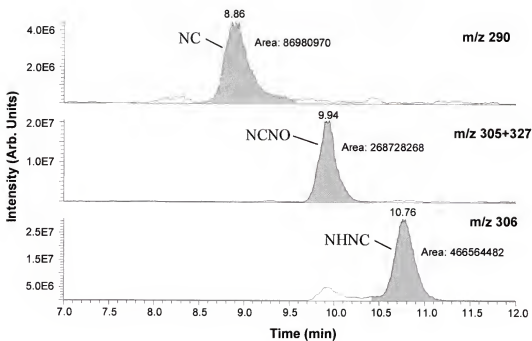


Figure 5-22: LC/MS results following LLX of norcocaine nitroxide from 50 mM TRIS. Prior to analysis, the reconstituted solution was filtered through a 0.45 μ m nylon filter.

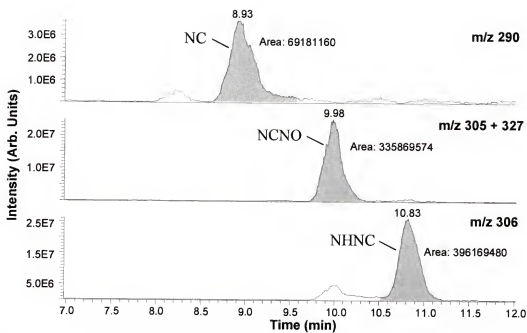


Figure 5-23: LC/MS results following LLX of norcocaine nitroxide from 25 mM K_2HPO_4/NaH_2PO_4 . Prior to analysis, the reconstituted solution was filtered through a 0.45 μm nylon filter.

Table 5-4: Area ratios of N-oxidative metabolites following LLX of NCNO from different buffered solutions. Values are calculated from LC/MS data in Figures 5-21, 5-22 and 5-23. The use of a Teflon filter versus a nylon filter has a large effect on the recovery efficiency of norcocaine nitroxide.

Area Ratio	NH ₄ OAc/Teflon	TRIS/Nylon	Phosphate/Nylon
NCNO/NC	0.871	3.090	4.855
NCNO/NHNC	0.154	0.576	0.848
NHNC/NC	5.657	5.364	5.727

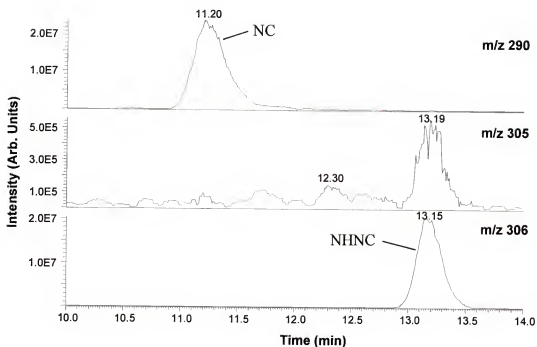


Figure 5-24: LC/MS results following SPE of NC, NCNO and NHNC. Prior to analysis, the reconstituted solution was filtered through a Teflon filter, which, as demonstrated in Table 5-4, reduces the recovery of norcocaine nitroxide, which may be the peak at 12.30 min.

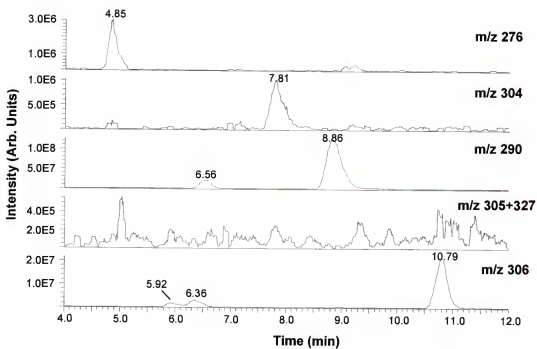


Figure 5-25: LC/MS of 100 μ M norcocaine *in vitro* incubation. Recovery of the metabolites was accomplished by liquid/liquid extraction. For designations of the metabolites, see Table 5-5.

Table 5-5: LC/MS/MS results of the 100 μ M norcocaine *in vitro* incubation.

Ret. Time (min.)	Precursor m/z	Major Product Ions	Designation
4.85	276	136, 154	Benzoylnorecgonine
5.92	306	136, 168	(m,p)-Hydroxynorcocaine
6.36	306	136, 168	(m,p)-Hydroxynorcocaine
6.56	290	258, 272	Artifact (?)
7.81	304	182	Cocaine
8.86	290	136, 168	Norcocaine
10.79	306	152, 184	N-hydroxynorcocaine

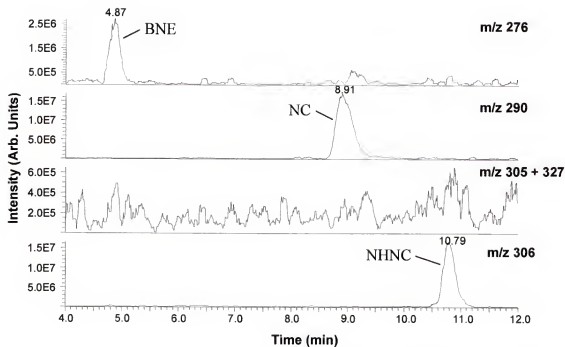


Figure 5-26: LC/MS of 100 μ M NHNC *in vitro* incubation following LLX. Based on the ion chromatogram for NCNO diagnostic ions (m/z 305 + 327), the formation of the hepatotoxic norcocaine nitroxide cannot be confirmed (expected t_R = 9.9-10.1 min.).

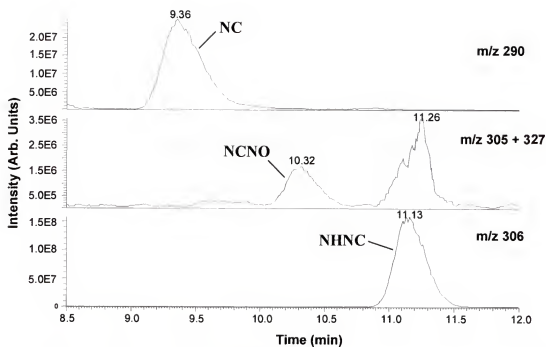


Figure 5-27: LC/MS following SPE of norcocaine nitroxide at an initial concentration of 100 μ M in 25 mM phosphate buffer.

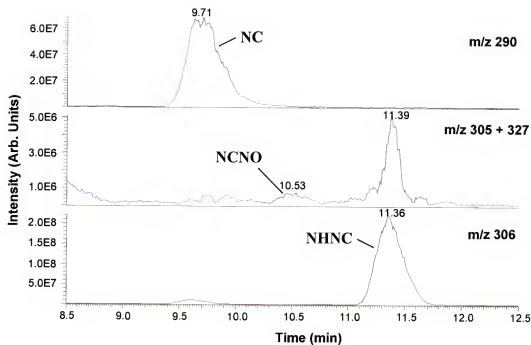


Figure 5-28: LC/MS following SPE of 100 μ M NCNO incubation with liver microsomes. Although the signal-to-noise is low, the peak at 10.53 min. is consistent with the retention time of norcocaine nitroxide.

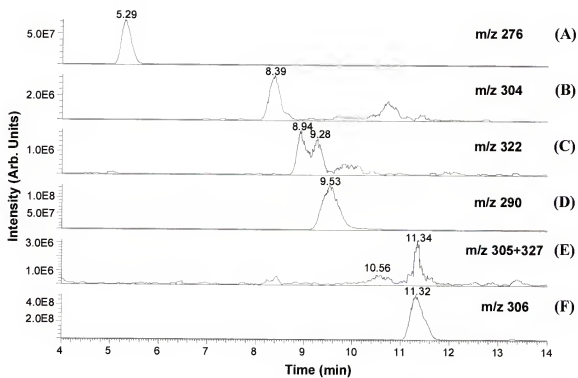


Figure 5-29: LC/MS of 500 μ M N-hydroxynorcocaine *in vitro* incubation following SPE. The minor peak at 10.56 min. is projected to be norcocaine nitroxide. Refer to the text and figures below for information on assignments of these metabolites.

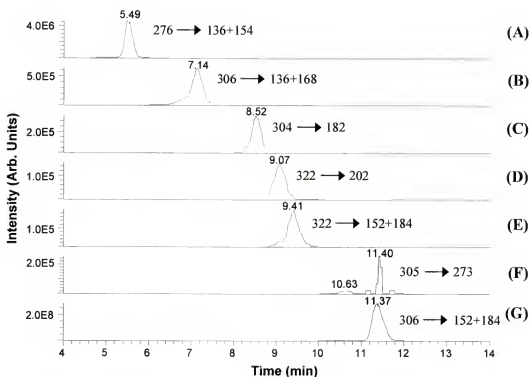


Figure 5-30: LC/MS/MS of selected metabolites from *in vitro* incubation of 500 μ M N-hydroxynorcocaine. The precursor-to-product ion transitions are given for each ion chromatogram. The peak at 10.63 min. corresponds to the MS/MS transition for norcocaine nitroxide, which is confirmed by its product ion spectrum (see Figure 5-31F). The narrow peak at 11.40 min. is due to a noise spike at m/z 273 during the MS/MS acquisition.

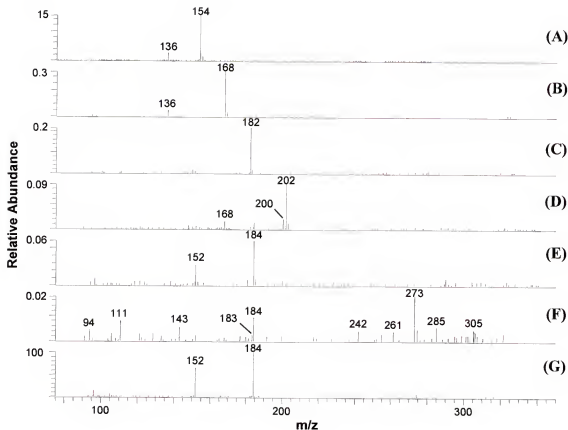


Figure 5-31: Product ion spectra from LC/MS/MS of 500 μM *in vitro* incubation of NHNC. The designated spectra (A-G) correlate to the eluting peaks from Figure 5-30A-G. The product ion spectrum in Figure 5-31F contains the structure specific ions at m/z 273 and 183 for NCNO.

Table 5-6: LC/MS/MS results of the 500 μ M NHNC *in vitro* incubation. Refer to Figures 5-30 and 5-31 for the retention times and product ion spectra for these metabolites.

Metabolite	Precursor m/z	Major Product Ions	Designation
A	276	136, 154	Benzoylnorecgonine
B	306	136, 168	(m,p)-Hydroxynorcocaine
C	304	182	Cocaine
D	322	202, 168	Unknown
E	322	152, 184	(m,p)-Hydroxy-N-hydroxynorcocaine
F	305	273	Norcocaine nitroxide
G	306	152, 184	N-hydroxynorcocaine

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

The growing demand within the biological sciences to gain a better insight into the fundamental processes of proteomics, pharmacology and toxicology has required analytical chemists to continuously reevaluate and improve the speed, sensitivity and selectivity of instrumentation and methodology to address these specific applications. This dissertation partially addressed these needs by assembling a new, versatile capillary LC/MS/MS system with high sensitivity and by developing new LC/MS/MS methodology to selectively analyze elusive, toxic metabolites of cocaine.

The newly developed QITMS, due to the advanced instrument software, is not constrained to the original parameters for which it was designed, unlike many of the commercially available mass spectrometers today. That is evident by the fact that this new QITMS was previously a GC/MS instrument with an EI/CI source. However, the hardware and software on the QITMS were modified such that this instrument is now capable of conducting LC/MS analyses with an ESI source. Furthermore, the mass range of the QITMS was extended from the GCQ default of 1000 u to 1800 u , and the octopole ion guide is under computer control, allowing proper ion gating from the ESI source to the QIT. A couple of other key operational changes made to the QITMS during the course of the dissertation project include ramping the main RF voltage during ion injection, m/z normalized resonant excitation amplitude for MS/MS, and dual MS/MS

monitoring. These modifications were imperative to achieve the successful development of this cLC/MS/MS system.

Improvement in the overall sensitivity of the QITMS is partially attributed to the development of the μ ESI interface. By taking advantage of the intrinsic property of ESI-MS as a concentration-sensitive detector, reduction of the mass flow to the ESI source maximizes the sensitivity of the QITMS. As demonstrated in Chapter 2, application of the μ ESI source in single-stage MS and MS/MS modes yielded adequate data with the consumption of a few tens of attomoles (10^{-17} moles) of the peptide angiotensin I at concentrations of 10 nM and 1 nM, respectively.

To further improve the sensitivity as well as increase the selectivity of the system for analyzing complex mixtures, cLC was integrated to the μ ESI-QITMS instrument. Nanobore capillary columns with I.D.s of 50 μ m were prepared directly in the same fused silica capillary as the μ ESI emitter to limit the amount of post-column volume, which could lead to extracolumn band broadening. Reversed-phase LC with gradient elution of a standard peptide mixture and of a tryptic digest of triose phosphate isomerase verified the enhanced selectivity of chromatographically separating the numerous components prior to MS detection. Additionally, improved concentration detection limits were realized via the preconcentration effect. Loading large sample volumes (e.g., 5 μ L) onto the cLC column yielded net elution concentration increases of approximately 100-fold. While the concentration sensitivity gains were marginal in single-stage MS mode, the application of cLC with tandem MS achieved significant improvements, as angiotensin I

was observed at a concentration of 10 pM (attomoles per microliter) and a mass loading of 50 attomoles.

Application of the cLC/MS/MS system to the analysis of extracts from the organism *Caenorhabditis elegans* for FMRFamide-like peptides yielded mixed results. A large number of components were observed in these samples via cLC/MS and cLC/MS/MS, yet only one of the targeted FLPs was identified and its verification is equivocal. Conversely, the standard peptide angiotensin I, which was spiked into the *C. elegans* extract prior to analysis, was detected within this complex sample at the level of 83 femtomoles and 335 attomoles during cLC/MS and cLC/MS/MS acquisitions, respectively. The observation of this internal standard at these amounts validates the developed instrument's sensitivity and the analytical methodology to identify trace-level compounds in "real-world" samples.

Identification of toxic species within biological systems is a major requisite in the areas of pharmacology and toxicology. Compounds which cause damage to living tissue need to be identified, often at trace levels, so that steps can be taken to counteract or eliminate the effects of these toxicants. With this in mind, a new LC/MS/MS method was developed to assay the suspected hepatotoxic metabolite of cocaine, norcocaine nitroxide.

The elusive nature of norcocaine nitroxide had made identification of this radical species problematic; however, under carefully controlled conditions, norcocaine nitroxide can be analyzed by LC/MS and LC/MS/MS. Using mobile phases buffered with ammonium acetate near pH 7, effective separation of norcocaine nitroxide and the closely related N-hydroxynorcocaine is achieved. It was necessary to use ammonium acetate

buffered solutions to inhibit the reduction of norcocaine nitroxide to N-hydroxynorcocaine, which readily occurs in acidic solutions and apparently by the free silanol sites of silica based stationary phases. Switching to a polyvinyl alcohol-based C₁₈ column did not completely alleviate the norcocaine nitroxide reduction reaction as it passed through the column, indicating that the weakly acidic polyvinyl alcohol protons may be sufficient to catalyze the reduction of NCNO to NHNC. Nevertheless, the polymeric C₁₈ column did significantly decrease the amount of peak tailing of norcocaine caused by secondary analyte/stationary phase interactions.

With an LC/MS/MS method for the N-oxidative metabolites of cocaine established, *in vitro* incubations of norcocaine and N-hydroxynorcocaine using rat liver microsomes were conducted. The LC/MS/MS results from the NC *in vitro* experiments did not demonstrate the presence of norcocaine nitroxide, but did show other oxidative metabolites, including N-hydroxynorcocaine. Also, the initial *in vitro* incubation of NHNC at 100 μ M did not provide evidence for the production of NCNO by LC/MS/MS. Repeating the incubation of N-hydroxynorcocaine in liver microsomes at 500 μ M and purifying the recovered metabolites with solid phase extraction, the biotransformation product norcocaine nitroxide was observed by LC/MS and LC/MS/MS, albeit at levels close to the detection limit for the established method.

The research presented in this dissertation sets the foundation for future studies in the areas of QITMS instrumentation and applied bioanalytical mass spectrometry. Advances and improvements in instrument development, analytical methodology and sample preparation can be envisioned to further enhance the accomplishments and overcome the setbacks of this project.

One of the key areas of increasing analytical feasibility of any system is improving the sensitivity of the instrumentation. An instrumental modification recently advanced to improve ion transmission in the intermediate pressure region of an ESI source is termed the ion funnel.^{239,240} A series of electrical lenses with narrowing internal diameters is arranged between the low-pressure side of the heated capillary and the multipole ion guide. The applied AC and DC potentials to the lens stack focuses the ion plume in the jet expansion region^{97,98} to a tight beam for transmission into the high vacuum region of the mass spectrometer. Reported improvements in overall ion transmission have been on the order of three-fold to ten-fold.²⁴⁰ Implementation of the ion funnel on the QITMS developed here may decrease mass detection limits into the high zeptomole (i.e., 10^{-19} moles) region.

Another means of improving the sensitivity of the mass spectrometer is to reduce the level of the chemical noise. A thorough study into the sources of the ubiquitous chemical background was not undertaken; however, some likely possibilities include the polypropylene sample tubes, the various connectors and fittings in direct contact with the mobile phase and the HPLC solvents. Extensive rinsing of the items in the flow stream and the use of higher-quality solvents may greatly improve the signal-to-noise of the QITMS data and further enhance the instrument's overall sensitivity.

The tendency of peptides to adhere to the container in which they are held may partially explain the inability to observe the FLPs from the *C. elegans* extracts. Therefore, revising the method of sample injection such that it is accomplished on-line with the cLC/MS apparatus may decrease sampling losses. This would require obtaining

a specialized injection valve and the reduction of extracolumn volume, yet the benefits should outweigh the costs.

An instrumental modification that may only provide a marginal improvement in sensitivity, yet would certainly make optimization of the μ ESI parameters vastly simplified, is the incorporation of visualization equipment. The addition of a CCD camera in the proximity of the heated capillary inlet would permit the user to visually inspect the condition of the μ ESI emitter for verification of a stable electrospray and for reproducible optimization of the emitter's position. This technology is readily available, reasonably inexpensive, and would require only minimal altering of the existing μ ESI interface to be adapted to the instrument, giving the user another means of monitoring the system in real-time.

Analytical applicability of the developed instrumentation can also be expanded by increasing the selectivity of analyses. One such possibility is the employment of an additional dimension of chromatography. For example, using ion exchange chromatography prior to reversed-phase chromatography would yield an independent means of selecting analytes, greatly enhancing the sample capacity of the system.¹⁷³ Such a setup would require additional switching valves and pumping capacity and would take significant time to optimize the different chromatographic conditions necessary for execution. However, once realized, the ability to conduct LC/LC/MS/MS would certainly be one of the most analytically informative and powerful instruments available.

Along similar lines is the use of immunoprecipitation methods for the preconcentration of selected compounds.²⁴¹ The utilization of antibodies for the specific selection of antigens is a well-established practice in the biological sciences and is the

basis of radioimmunological assays (RIA). Antibodies raised the peptide FMRFamide are commercially available and have been used to isolate other FLPs.^{187,242} It would be advantageous to employ these antibodies in the extraction process to selectively capture the FLPs from *C. elegans* prior to cLC/MS/MS analyses in order to greatly simplify the sample mixtures and to facilitate the identification of these neuropeptides.

Some other improvements in methodology that would likely contribute to the overall improvement of the analytical capabilities of the newly constructed cLC/MS/MS system include the creation of a data-dependent scan function and the development of an escalated LC gradient. The utility of the Visual Basic software that drives the QITMS is that other mass spectrometric functions can be created within the VB code, as demonstrated with the dual MS/MS monitoring routine. Should the chemical background in the cLC/MS data be adequately reduced (*vide infra*), the data-dependent scanning routine could be engaged to select the most abundant ion for each eluting peak for MS/MS data analysis. Therefore, this function would provide structural information on a wider range of eluting components. Conversely, an escalated gradient, in which the rate of the linear ramp is increased with time, would better optimize the chromatographic separation efficiency of compounds with a wide variety of polarities. For example, a gradient ramp at the beginning of an LC run could be at 4% per minute to allow adequate separation of the hydrophilic compounds (during reverse-phase LC) and the gradient could escalate to 5% then to 6%, *et cetera*, to prevent band broadening of hydrophobic analytes. It is hypothesized that this gradient method would provide adequate chromatographic resolution across the wide range of solvent strengths employed in this dissertation research to observe the peptides with varying lengths and polarities.

The development of this QITMS with eLC and with the μ ESI interface has demonstrated the ability to explore biological problems while still having the freedom to be adapted to meet the challenge of other analytical chemistry problems. Whether in the area of the biological, environmental or forensic sciences, this research-grade QITMS provides an effective platform to conduct future research.

With the foundational LC/MS/MS method established for analyzing the toxic metabolites of cocaine, numerous experiments can be envisioned to improve the method and better characterize these metabolites, particularly norcocaine nitroxide. One of the primary objectives that requires addressing is the obviously low sensitivity for NCNO. Based on the results presented in Chapter 5, norcocaine nitroxide seems to bind strongly to Teflon, which was discovered to be contained in the membranes of the SPE cartridges used. Obtaining a different brand of SPE devices without Teflon should greatly improve the recovery of NCNO for future *in vitro* experiments. Another possibility that may enhance the sensitivity for NCNO is to employ an LC column packed with polystyrene divinylbenzene (SDB) particles. The silica-based and polyvinyl alcohol-based columns showed some indication that norcocaine nitroxide was being reduced to N-hydroxynorcocaine as NCNO partitioned through these columns. If this reaction is indeed being catalyzed by the free acidic protons of the unmodified stationary phase of the silica and polyvinyl alcohol columns, the lack of such acidic protons on the SDB stationary phase should eliminate the conversion of NCNO to NHNC.

Research presented here and elsewhere²¹⁴ has indicated that NCNO binds irreversibly to microsomal proteins. In order to better assess whether this is the case and to what degree this binding occurs, a highly purified NCNO will be required, preferably

one that is isotopically labeled (e.g., ^2H , ^3H). A pure norcocaine nitroxide standard will allow accurate quantitative analyses by LC/MS/MS so that the percent loss of NCNO following *in vitro* incubation can be determined. The use of a radiolabeled standard would permit the tracking of the NCNO by alternate means such that it could be located for identification. For example, irreversible binding of radiolabeled NCNO to a protein in the liver could be isolated and that tagged protein could be identified by enzymatic digestion and LC/MS/MS. This process could lead to specific knowledge as to the cause of norcocaine nitroxide's hepatotoxicity.

After further characterization of NCNO *in vitro* to ascertain the percentage of protein binding and to determine the efficiency of biotransformation to norcocaine nitroxide, *in vivo* experiments could be conducted. Research has demonstrated that cocaine abuse leads to liver damage,²⁰⁸ which is believed to be caused by NCNO; however, norcocaine nitroxide has yet to be identified following *in vivo* dosing of cocaine at toxic levels. While it is difficult at this stage to predict whether NCNO could be isolated from biological matrices after *in vivo* experiments, the LC/MS/MS method developed here with subsequent improvements described above may help expand the understanding of the toxicity of the N-oxidative metabolites of cocaine.

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BIOGRAPHICAL SKETCH

Kevin Joseph McHale was born on July 28, 1972 to Joseph and Beverly McHale. Raised in McDonald, OH, a small village north of Youngstown, OH, Kevin enjoyed backyard football, collecting baseball cards, and playing video games with his childhood friends.

During his youth, Kevin aspired to be either a professional basketball player or an astronaut. Unfortunately, his lack of physical attributes prohibited a career in the NBA and poor eyesight precluded him from service in NASA. Nevertheless, Kevin's interest in science (and science fiction) never wavered. In high school, he excelled in chemistry, physics and calculus, obtaining several recognitions in these areas, including second prize in a local physics olympics event.

After graduating as co-valedictorian from McDonald High School in 1990, Kevin pursued a Bachelor of Science degree in chemistry at the University of Akron. His interest in advanced chemistry research caught the attention of one of the newer professors in the chemistry department, Dr. Chrys Wesdemiotis. An invitation to join the Wesdemiotis research group was extended to Kevin during his sophomore year, which he eagerly accepted.

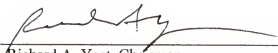
Working closely with the graduate students in Dr. Wesdemiotis's group, Kevin began to understand not only the fundamentals of mass spectrometry but also the premises of independent research in chemistry. This led to the ambitious endeavor of examining the gas-phase chemistry of buckminsterfullerene using tandem mass

spectrometry. While Dr. Wesdemiotis had reservations about the success of the proposed research, he encouraged Kevin to explore the project. Eventually, the initiative was fruitful, as the research results provided new insight into the chemistry of buckminsterfullerene.

Although encouraged to continue his research training in a doctoral program, Kevin decided to broaden his experience in an industrial setting. Obtaining his B.S. degree in 1994, Kevin accepted a position in the mass spectrometry lab at the DuPont Merck Pharmaceutical Company. Excited about the prospects of applying his knowledge and research experience in a “real-world” environment, he quickly discovered that research in academia and industry are very different. While a vast amount of practical knowledge and experience was gained, Kevin soon became disenchanted with the prospects of attaining his career goal of directing applied research projects. Therefore, in the fall of 1997 Kevin left DuPont Merck to pursue a Ph.D. at the University of Florida.

Under the direction of Dr. Richard Yost, Kevin was given the opportunity and freedom to explore many avenues of biological research using mass spectrometry. The encouragement and assistance of Dr. Yost and his graduate students, both past and present, helped hone Kevin’s research skills and broaden his scope of knowledge in analytical chemistry. Upon receiving his Ph.D., Kevin will start his new position as an LC/MS Field Marketing Specialist at ThermoFinnigan in Piscataway, New Jersey.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Richard A. Yost, Chairman
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Robert T. Kennedy
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



David H. Powell
Scientist of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Steven A. Benner
Distinguished Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Sheldon M. Schuster
Professor of Biochemistry and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2002

Dean, Graduate School